INSTITUTO SUPERIOR DE AGRONOMIA
Universidade de Lisboa

BRIDGING THE GAP TO NONTOXIC FUNGAL CONTROL

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Orientadora: Doutora Sara Alexandra Valadas da Silva Monteiro

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Tese Elaborada para Obtenção do Grau de Doutor em Biologia

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Abstract

Blad is an edible, *Lupinus* cotyledon-derived 173 amino acid residue polypeptide which occurs naturally and undissociated as part of a soluble 210 kDa oligomer, hereby termed Blad-containing oligomer (BCO). BCO is isolated during growth of *Lupinus* seedlings and is a breakdown product of β-conglutin catabolism. In this work, BCO inherent, potent and wide spectrum antifungal activity was demonstrated under *in vitro* and greenhouse conditions. Several field trials showed that BCO exhibits an equal or better performance than the conventional chemicals over a large variety of crops/fungal pathogens. BCO antifungal activity was also showed to extend to human pathogens, including both yeasts and filamentous fungi. *In vitro* tests demonstrated a higher efficacy on a molar basis when compared to the best chemical fungicides tested, with an absence of mammal toxicity or genotoxicity. In addition to the lectin and catalytic activities which allow BCO to target the fungal cell wall, this work revealed other highly complex and multitarget mode(s) of action. BCO enters fungal cells causing several morphological alterations and seems to essentially act through inhibition of metal ion homeostasis resulting in an apoptotic cell death. In an attempt to provide a structural characterization of Blad, data from electronic microscopy, dynamic light scattering, SAXS and X-ray crystallography were obtained from heterologous expressed Blad, however the results were not conclusive. Last but not least, this work also aimed at the development of a "super-Blad": a fusion protein consisting of Blad and two selected antibacterial peptides, SP10-5 and Sub5. The resulting chimeras not only maintained a strong antibacterial activity but were also able to retain the ability to inhibit the growth of both yeast and filamentous fungi.

In summary, the overall approach of this thesis considered phytopathogens and human pathogens as belonging to the same scientific area and therefore aimed at producing a uniform natural multitarget, step-stone antifungal solution.

Key-Words: Blad, Blad-containing oligomer, antifungal activity, mode of action, fusion proteins

Resumo

A Blad é um fragmento interno com 173 aminoácidos da maior proteína de reserva de sementes de plantas do género *Lupinus*, a β-conglutina, e ocorre naturalmente como parte de um oligómero solúvel de 210 kDa denominado de oligómero que contém a Blad – BCO – do inglês *Blad-Containing Oligomer*. Neste trabalho, foi demonstrado o vasto e potente espetro de atividade antifúngica do BCO, tanto *in vitro* como *in vivo*, contra vários fungos fitopatogénicos, sendo esta igual ou superior à dos fungicidas químicos convencionais. Demonstrou-se também que a sua atividade antifúngica se estende a fungos unicelulares com relevância clínica e os testes de suscetibilidade realizados revelaram novamente uma maior eficácia do que a dos melhores fungicidas químicos, com ausência de toxicidade e genotoxicidade em células mamíferas. Além das atividades de lectina e catalíticas que permitem que o BCO tenha como alvo a parede celular dos fungos, este trabalho revelou outro(s) modo(s) de ação complexo(s). O BCO entra nas células de fungos provocando várias alterações morfológicas e parece atuar essencialmente através da destabilização da homeostase de iões metálicos, culminando numa morte celular por apoptose. Com o objetivo de obter a estrutura tridimensional da Blad recorreu-se à sua expressão heteróloga seguida de estudos de microscopia eletrónica, DLS, SAXS e cristalografia de raios-X, mas, no entanto, os resultados obtidos não foram conclusivos. Por fim, este trabalho pretendeu desenvolver uma "super-Blad": uma proteína de fusão constituída pela Blad e por dois péptidos antibacterianos, SP10-5 e Sub5. A proteína de fusão obtida manteve não só uma forte atividade antibacteriana como reteve a capacidade de inibir o crescimento tanto de leveduras como de fungos filamentosos.

Em suma, a abordagem geral desta tese considerou os fungos fitopatogénicos e os patogénicos humanos como pertencendo à mesma área cientifica e, como tal, teve como objetivo produzir uma solução antifúngica uniforme, natural, inovadora e *multitarget*.

Palavras-chave: Blad, BCO, atividade antifúngica, modo de ação, expressão, proteínas de fusão

Resumo alargado

A resistência dos fungos patogénicos a agentes antifúngicos é considerada, hoje em dia, um grave problema tanto na agricultura como na saúde humana. Ao desenvolverem resistência a um fungicida específico, as populações de fungos tornam-se automaticamente resistentes a outros compostos que tenham um mecanismo de ação semelhante. No caso da agricultura, o desenvolvimento de antifúngicos sistémicos agravou drasticamente este problema e, no caso das infeções na saúde humana, o problema das resistências é também agravado por haver um número muito limitado de classes de moléculas com diferentes modos de ação. O uso continuado e, por vezes, indiscriminado, de agentes antifúngicos também tem contribuído para o aparecimento de resistências. Além disto, a aplicação massiva de fungicidas na agricultura tem tido como consequência o aparecimento de novas resistências em fungos patogénicos que também causam doenças na área clínica. Paradoxalmente, um número alarmante de novas moléculas estrutural ou farmacologicamente semelhantes a fármacos previamente aprovados para serem usados na área clínica têm obtido aprovação para serem usadas como antifúngicos agrícolas.

Neste contexto, há uma necessidade extrema de novos agentes antifúngicos, com novos modos de ação, quer para a agricultura quer para a área clínica. Preferencialmente, estes novos agentes não deverão promover o desenvolvimento de resistências. Por exemplo, antifúngicos com um mecanismo de ação *multi-target* estão descritos como sendo menos propícios de promoverem o desenvolvimento de resistências nos microrganismos. No entanto, presentemente, a maioria dos antifúngicos disponíveis tem como alvo uma proteína ou uma via metabólica específica do agente patogénico, o que promove o rápido desenvolvimento de resistências.

Estudos prévios realizados no Instituto Superior de Agronomia, conduziram à descoberta de um polipéptido com potencial antifúngico denominado Blad. A Blad é um fragmento interno com 173 aminoácidos da maior proteína de reserva das sementes de plantas do género *Lupinus*, a β-conglutina, e acumula-se como um oligómero de 210 kDa (denominado de oligómero que contém a Blad – BCO – do inglês *Blad-Containing Oligomer*) nos cotilédones entre o 4° e o 12° dia após o início da germinação.

Na primeira fase do trabalho, foram estudadas as principais propriedades bioquímicas do BCO, tendo-se verificado que este apresenta atividades catalíticas de β-*N*-acetil-D-glucosaminidase,

de quitosanase e de lectina. Os resultados demonstraram ainda que a atividade lectina do BCO se deve exclusivamente à Blad. Ficou também demonstrada a sua elevada afinidade para a quitina e, tendo em conta que tanto a quitina como a quitosana são constituintes importantes da parede celular dos fungos filamentosos, o passo seguinte passou por avaliar a sua atividade antifúngica. Os testes de suscetibilidade realizados revelaram que o BCO possui uma elevada atividade antifúngica, tanto *in vitro* como *in vivo*, contra vários fungos patogénicos, sendo esta igual ou superior à atividade demonstrada pelos fungicidas químicos comercialmente disponíveis. Nesta fase, foi necessário separar os resultados relevantes para a sua aplicação na agricultura (prosseguindo com a sua validação em ensaios de campo) dos resultados relevantes para a área clínica.

Neste trabalho, foram apenas estudados fungos unicelulares(leveduras) com relevância clínica por duas razões: são os que maior prevalência têm para a saúde humana e são universalmente aceites como modelos dos fungos filamentosos. Desta forma, foi possível estudar o modo de ação do BCO recorrendo a leveduras. Ensaios de imunofluorescência e de *imunogold* efetuados em *Candida albicans* revelaram que o BCO tem a capacidade de atravessar o envelope celular e entrar nas células, tornando-as metabolicamente inativas, não viáveis e não cultiváveis. Foi também visível a perda de integridade da membrana celular, mas sem danos visíveis ao nível da parede. Ainda não foi possível esclarecer o mecanismo específico usado pelo BCO para entrar nas células, ficando apenas determinado que este é independente do ergoesterol. Foram também observadas várias alterações ultraestruturais nas células na presença do BCO, nomeadamente, espessamento da parede celular e aumento do volume celular. Uma análise global ao transcriptoma de *C. albicans* e os ensaios de haploinsuficiência permitiram determinar que o ambiente adverso intra e/ou extracelular provocado pelo BCO é resultado daquilo que foi considerado ser o seu mecanismo de ação primário – a capacidade de sequestrar catiões metálicos, com particular efeito no zinco e ferro, levando à perda da homeostase celular. Como consequência, a célula entra em *stress* oxidativo e produz Espécies Reativas de Oxigénios (EROs), culminando numa morte celular por apoptose.

Para avaliar uma possível aplicação do BCO como agente antifúngico para o tratamento de micoses tópicas, realizou-se uma análise preliminar da sua geno- e citotoxicidade em mamíferos. Os resultados obtidos, quer *in vitro*, quer *in vivo*, foram bastante promissores, uma

vez que não revelaram qualquer evidência de toxicidade em células mamíferas, tanto após exposições agudas como de curta duração.

Numa segunda fase do trabalho, recorreu-se à produção da Blad recombinante, através da sua expressão heteróloga em *Escherichia coli.* Como já foi referido, a Blad é uma proteína de 20 kDa e corresponde à maior fração de um oligómero de 210 kDa, o BCO, não ocorrendo isolada de forma natural. Assim, recorremos à sua produção sob a forma recombinante com o objetivo principal de determinar a sua estrutura tridimensional. Apesar da sequência de aminoácidos da Blad ser maioritariamente composta por aminoácidos hidrofílicos, esta é bastante insolúvel após expressão heteróloga em *E. coli,* exibindo um comportamento semelhante ao das moléculas hidrofóbicas. Uma possível abordagem para ultrapassar este problema passa por recorrer a parceiros promotores de solubilidade – proteínas que, tal como o nome indica, após serem ligadas à proteína-alvo promovem o seu correto *folding.* Outra abordagem possível é substituir aminoácidos hidrofóbicos por hidrofílicos e/ou cortar a sequência da proteína o máximo possível, sem interferir com a sua atividade biológica. De modo a maximizar as hipóteses de obter a proteína sob a forma solúvel, foi realizada uma triagem onde se testaram, simultaneamente: cinco formas truncadas da Blad e/ou com alterações na sequência de aminoácidos, juntamente com a sua sequência original; três parceiros promotores de solubilidade *(maltose-binding protein (MBP), N-utilization substance protein A (NusA) e Thioredoxin (Trx));* três estirpes de *E. coli;* e dois meios de cultura diferentes. A combinação que apresentou resultados mais promissores foi a construção com MBP (His_6-MBP-Blad), tendo sido selecionada para os ensaios seguintes. No entanto, após ensaios preliminares de caraterização da estrutura (microscopia eletrónica, espalhamento de luz dinâmico e espalhamento de raio-x a baixo ângulo) e dos ensaios de cristalografia de raios-X foi identificada uma contaminação com uma proteína de *E. coli* – chaperonina GroEL. A contaminação com chaperonas do hospedeiro durante a expressão heteróloga é um problema comum, agravado neste caso pelo facto de tanto a construção His_6-MBP-Blad como a proteína contaminante apresentarem o mesmo peso molecular, dificultando a sua deteção. Após várias tentativas de eliminar a contaminação com GroEL, esta persistiu.

Por último, e na sequência da produção da Blad sob a forma recombinante, iniciou-se o desenvolvimento de um novo biopesticida – uma proteína híbrida constituída por duas moléculas com propriedades antimicrobianas complementares (antifúngica e antibacteriana).

Para esse efeito, fundiu-se o gene que codifica a Blad com o gene de dois péptidos antibacterianos – SP10-5 e Sub5. Desta forma desenvolveu-se uma "super-Blad": uma proteína de fusão com propriedades antifúngicas e antibacterianas. As atividades antibacteriana e antifúngica das proteínas de fusão foram testadas contra um conjunto de bactérias, leveduras e fungos filamentosos e os resultados obtidos foram bastante promissores, principalmente para a fusão da Blad com o péptido SP10-5, onde foi possível preservar a atividade antibacteriana do péptido e a atividade antifúngica da Blad.

A Blad é, sem duvida, um polipéptido notável que parece preencher todos os pré-requisitos para funcionar como um novo paradigma ou até mesmo como um futuro protótipo para o controlo de fungos patogénicos. Esta tese pretende divulgar o enorme potencial e segurança de uma nova terapêutica tanto para a agricultura como para a saúde humana baseada no BCO. Atualmente o BCO é já o ingrediente ativo de um fungicida agrícola, Fracture™, à venda em alguns países (EUA, Canadá e Coreia do Sul) e em breve estará disponível noutras jurisdições (Europa, China e Austrália). O desenvolvimento da "super-Blad", uma proteína que apresenta simultaneamente atividade antifúngica e antibacteriana constitui uma importante inovação e pode representar uma nova abordagem para uso na agricultura. Além disto, a descoberta do novo e *multi-target* mecanismo de ação do BCO levou a que o *Fungicide Resistance Action Committee* (FRAC) o incluísse numa nova categoria, M12, do seu *Code List*© de 2016. No entanto, é preciso ainda compreender totalmente não só o modo exato como o BCO entra nas células, mas também averiguar a existência de toxicidade sistémica em células mamíferas e a sua estabilidade após administração intravenosa. Estes estudos irão permitir determinar o potencial uso do BCO no tratamento de infeções fúngicas sistémicas. Por último, e considerando que o conhecimento da estrutura tridimensional da Blad pode ser uma mais valia para o total conhecimento do seu modo de ação, é necessário, no futuro, seguir uma abordagem que permita produzir a Blad através de expressão heteróloga, solúvel, mas sem contaminações de proteínas do hospedeiro.

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General Introduction

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1.1 Fungi: a threat to animal, plant and ecosystem health

Infectious diseases caused by fungi are increasingly recognized as a worldwide hazard to food security (Pennisi, 2010; IOM, 2011). This is not a new problem since fungi have long been known to constitute a widespread threat to crops. Plant disease epidemics caused by fungi and the fungal-like oomycetes have altered the course of human history. In the 19th century, late blight led to starvation, economic ruin and the downfall of the English government during the Irish potato famine and, in the 20th century, Dutch elm blight and chestnut blight laid bare urban and forest landscapes. The threat of plant disease has not abated, in fact it has heightened by resource-rich farming practices and exaggerated in the landscape by microbial adaptation to new ecosystems, brought about by trade and transportation (Grunwald *et al*., 2008), and by climate fluctuations (Brown and Hovmoller, 2002; Anderson *et al*., 2004).

Pathogenic fungi have not been widely recognized as posing major threats to animal health. This perception is changing rapidly owing to the recent occurrence of several high-profile declines in wildlife caused by the emergence of previously unknown fungi (Daszak *et al*., 2000; Smith *et al*., 2006). For example, during March 2007, a routine census of bats hibernating in New York State revealed mass mortalities (Blehert *et al*., 2009). Within a group of closely clustered caves, four species of bats were marked by a striking fungus growing on their muzzles and wing membranes, and the name 'white nose syndrome' (WNS) was coined. After the initial outbreak, the ascomycete fungus *Geomyces destructans* was shown to fulfil Koch's postulates and was described as the cause of WNS in American bat species (Gargas *et al*., 2009; Lorch *et al*., 2011). Mortalities exhibiting WNS have subsequently been found in an increasing number of bats overwintering sites and, by 2010, the infection was confirmed to have emerged in at least 115 roosts across the United States and Canada, spanning over 1,200 km (Frick *et al*., 2010). Cases of this sort are no longer perceived to be atypical. The skin-infecting amphibian fungus *Batrachochytrium dendrobatidis* was discovered in 1997 (Berger *et al*., 1998) and named in 1999 (Longcore *et al*., 1999). *B. dendrobatidis* has been shown to infect over 500 species of amphibians in 54 countries, on all continents where amphibians are found (Fisher *et al*., 2009), and is highly pathogenic across a wide diversity of species. Studies using preserved amphibian specimens showed that the first appearance of *B. dendrobatidis* in the Americas coincided with a wave of population declines that began in southern Mexico in the 1970s and proceeded through Central America to reach the Panamanian isthmus in 2007 (Cheng *et al*.,

2011). As a consequence of the infection, some areas of Central America have lost over 40% of their amphibian species (Crawford *et al*., 2010), a loss that has resulted in measurable ecosystem-level changes (Colón-Gaud *et al*., 2009).

Fungal infections causing widespread population declines are not limited to crops, bats and frogs; studies show that they are emerging as pathogens across diverse taxa, including soft corals (for example, sea-fan aspergillosis caused by *Aspergillus sydowii*) (Kim and Harvell, 2004), bees (the microsporidian fungus *Nosema* sp. associated with colony collapse disorder) (Cameron *et al*., 2011), and as human and wildlife pathogens in previously non-endemic regions (for example, the emergent virulent VGII lineage of *Cryptococcus gattii* in northwest America (Byrnes III *et al*., 2010) and *Cryptococcus neoformans* across southeast Asia (Simwami *et al*., 2011)). Although the direct causal relationship is uncertain in some of these diverse host–pathogen relationships, it seems that pathogenic fungi are exerting a pronounced effect on the global biota.

1.2 Fungal infections in agricultural crops

According to the United Nations World Population Prospects (United Nations, 2015), the number of people in the planet will reach approximately 10 billion in 2050 and, according to Food and Agriculture Organization (FAO, 2016) the available farmland will decrease from the current 0.218 ha/capita to 0.181 ha/capita. The same study estimates a decrease on crop yields of around 17% by 2050. So, overall, a productivity increase on crop yields of around 60% will be required to feed the planet. The world's agriculture depends largely on a small fraction of the many thousands of plant species grown worldwide, so, intercontinental dispersal of pathogens may cause diseases of crops on a global scale (Brown and Hovmoller, 2002).

Plants constitute an excellent ecosystem for microorganisms because they offer a wide diversity of habitats, including the phyllosphere (aerial plant part), the rhizosphere (zone of influence of the root system) and the endosphere (internal transport system) (Montesinos *et al*., 2002; Ferreira *et al*., 2006). The increase of plant emerging infectious diseases is driven mainly by anthropogenic environmental changes (such as introductions, farming techniques and habitat disturbance) (Anderson *et al*., 2004) and the wind dispersal of fungal spores for hundreds or thousands of kilometers has caused the spread of several important diseases on a continental or global scale and allowed the regular reestablishment of diseases in regions

where the climate is seasonally unfavorable (Brown and Hovmoller, 2002). The risk of global spread of disease is also incremented by the limited genetic variability of many modern crops, as compared to that of related wild species or landrace crops (Brown and Hovmoller, 2002). Emerging infectious diseases caused by fungi are therefore increasingly recognized as presenting a worldwide threat to food security (Fisher *et al*., 2012).

1.2.1 Most common plant fungal pathogens

In plants, 120 genera of fungi, 30 types of viruses and 8 genera of bacteria are responsible for the ca. 11,000 diseases that have been described (Ferreira *et al*., 2006). Contrary to what happens with humans, the importance of the environment on the development of plant diseases has been known for over two thousand years (Ghini *et al*., 2008). Since environment and diseases are closely related, climate change is altering the geographical and temporal distribution of phytosanitary problems (Ghini *et al*., 2008). Nevertheless, a recent study compiled the top 10 fungal pathogens in plant pathology, where the fungus with the strongest appearance was *Magnaporthe oryzae* followed by *Botrytis cinerea* (Dean *et al*., 2012).

M. oryzae (anamorph *Pyricularia oryzae)* is the Ascomycete fungus responsible for rice blast, the most damaging rice disease worldwide of cultivated rice (Saleh *et al*., 2014) and is considered a major threat to global food security (Giraldo *et al*., 2013). Damage to the rice plants results from either leaf blast, which kills or debilitates seedlings, or neck or panicle blast, which destroys the rice grain during the seed-setting stage (Tajul *et al*., 2012). Blast disease was first reported in the United States in 1876 and was identified in 85 rice-producing countries or regions worldwide (Wang *et al*., 1876). It is estimated that every year an amount of rice enough to feed 60 million people is destroyed by this disease (Tani *et al*., 2005). The major mechanisms of disease spread over short and long distances are airborne inoculum dispersal and dissemination of infected seeds, respectively.

In the last decade, *M. oryzae* has also been associated with blast disease on barley, rye, and triticale in areas of central-western and southern Brazil. Because wheat blast has not yet been detected in North America, *M. oryzae* is considered a major quarantine pathogen and a possible threat to wheat crops in the United States (Castroagudín *et al*., 2014).

B. cinerea has a broad host range, comprising more than 200 crop species worldwide (Baarlen *et al*., 2007). It is most destructive on mature or senescent tissues of dicotyledonous hosts, but it usually gains entry to such tissues at a much earlier stage in crop development and remains quiescent for a considerable period before rapidly rotting tissues when the environment is conducive and the host physiology changes (Williamson *et al*., 2007). *B. cinerea* is difficult to control because it has a variety of modes of attack, diverse hosts as inoculum sources, and it can survive as mycelia and/or conidia, or for extended periods, as sclerotia, in crop debris (Williamson *et al*., 2007). It is responsible for a very wide array of symptoms and these cannot easily be generalized across plant organs and tissues. Soft rots, accompanied by collapse and water soaking of parenchyma tissues, followed by a rapid appearance of grey masses of conidia are perhaps the most typical symptoms on leaves and soft fruits (Williamson *et al*., 2007).

Among the other fungi ranked as the top 10 plant pathogens are *Puccinia* spp., *Fusarium graminearum* and *F. oxysporum,* and *Colletotrichum* spp. (Dean *et al*., 2012). *Puccinia* spp. are obligate biotrophic basidiomycetes responsible for the three rust diseases on wheat, possibly the most feared diseases in most wheat-growing regions of the world (Leonard and Szabo, 2005). *F. graminearum* is a highly destructive pathogen of all cereal species and is the most common causal agent of *Fusarium* head blight (FHB) in North America and many other parts of the world (Goswami and Kistler, 2004). *F. oxysporum* causes vascular wilt on a wide range of hosts, namely tomato, melon, banana, cotton and chickpea (Michielse and Rep, 2009). *Colletotrichum* is one of the most common and important genera of plant pathogenic fungi. Virtually every crop grown throughout the world is susceptible to one or more species of *Colletotrichum*. These fungi cause anthracnose spots and blights of aerial plant parts and post-harvest rots. Members of this genus cause major losses to economically important crops, especially fruits, vegetables and ornamentals (Dean *et al*., 2012).

Worryingly, the battle lines between fungal pathogens and plants are constantly expanding, mostly as a result of human activity, with the emergence of new hypervirulent isolates and the spread of pathogens to new geographic areas. As just one example, the devastating wheat blast disease caused by *M. oryzae* was recently reported in Bangladesh (Callaway, 2016). This agricultural emergency is the first description of this disease in Asia and has serious implications for wheat production throughout the continent. Pathogen geographical expansion

is likely facilitated by global warming (Bebber *et al*., 2013), and of extreme concern is emergence of multi fungicide resistant crop-destroying fungi.

1.3 Fungal infections in humans

At the beginning of the 20th century, bacterial epidemics were a global and important cause of mortality but, in contrast, fungal infections were almost not taken into account (Vandeputte *et al*., 2012). Currently, among the various opportunistic pathogens, pathogenic fungi represent, worldwide, a serious and important threat for human's health (Denning and Hope, 2010; Arvanitis *et al*., 2014). Over the past two decades there was a significant increase of severe fungal infections due to opportunistic fungal pathogens (Bendaha *et al*., 2011; Kathiravan *et al*., 2012). However, this effect on human health is not widely recognized, and deaths resulting from these infections are often overlooked (Brown *et al*., 2012). For example, the World Health Organization has no program for fungal infections, and most public health agencies - with the singular exception of the U.S. Centers for Disease Control and Prevention (CDC) - conduct little or no mycological surveillance (Brown *et al*., 2012). Fungal outbreaks are more common than most people acknowledge, and reports of outbreaks caused by unusual fungal pathogens are increasing (Litvintseva *et al*., 2015). Annually, 1.5 to 2 million people die from fungal infections worldwide, which is more than from malaria or tuberculosis. Nevertheless, human fungal infections still receive less attention than viral or bacterial diseases, though the associated mortality rate is very high, often exceeding 50% (Jampilek, 2016).

The immune system of healthy individuals has effective mechanisms for preventing fungal infections, and the current incidence of invasive diseases is largely a result of substantial escalation in immunosuppressive infections over the last few decades, such as HIV/AIDS and modern immunosuppressive and invasive medical interventions (Mishra *et al*., 2007; Denning and Hope, 2010; Brown *et al*., 2012; Litvintseva *et al*., 2015). Under conditions of impaired immune responses or a rupture in host barriers, fungi are able to invade normally pathogen-free areas of the human body. Once there, they can cause severe infections that are difficult to recognize and to treat and are often ultimately lethal (Brown *et al*., 2012). On the other hand, fungal infections in intensive care units (ICUs) constitute a well-documented problem and a nosocomial threat in critically-ill patients (Bansal and Pande, 2013), mainly because ICUs carry all the risk factors for fungal infections – e.g. indwelling lines and catheters, broad spectrum

antibiotic cover, hemodialysis, total parenteral nutrition steroids and mechanical ventilation (Bansal and Pande, 2013). Very old age and preterm birth are also risk factors (Jampilek, 2016). Although the progress of medicine has helped to control infectious diseases, the advent of certain medical practices has actually favored the occurrence of other microbial infections (Del Poeta, 2010; Delarze and Sanglard, 2015).

In an attempt to effectively eliminate these infections, early diagnosis and species identification are of paramount importance, but identification at the species level is often not available in most microbiology laboratories until several days into the course of treatment (Andes, 2013). Isolation and identification of the infecting organisms are extremely important for the proper management of infections due to the less common opportunistic fungi (Pfaller and Diekema, 2004). Despite the significant advances over the last decade on detection, identification and analysis of fungal communities on mucosal surfaces (Huffnagle and Noverr, 2013), it is still undoubtedly true that current gold standards for invasive fungal infections diagnosis are lacking in both sensitivity and rapidity, thus delaying treatment and undermining the survival of patients at risk (Brown *et al*., 2012; Arvanitis *et al*., 2014; Maubon *et al*., 2014). Traditional diagnostic methods, such as histopathology and culture, which are still considered the gold standards, have low sensitivity and rapidity, which underscore the need for developing new means of detecting fungal infectious agents (Arvanitis *et al*., 2014). Epidemiological data for fungal infections are also notoriously poor because fungal infections are often misdiagnosed (Brown *et al*., 2012). Moreover, although it is well known that treatment is initiated too late in the majority of patients, identification of the optimal time-point to commence antifungal therapy remains unknown (Cornely *et al*., 2012).

Superficial infections of the skin and nails are the most common fungal diseases in humans, affecting approximately 25% of the population worldwide (Havlickova *et al*., 2008), followed by mucosal infections of the oral and genital tracts. Between 50 and 70% of women suffer from at least one episode of vulvovaginitis and 5 to 8% have at least four episodes annually (Sobel, 2007). Despite the much lower incidence of invasive fungal infections when compared to those involving skin or nails, they are of much greater concern due to their high rate of mortality (Deodato *et al*., 2016). Morbidity and mortality associated with these infections are also substantial, and there is no doubt that fungal diseases have emerged as an important public health problem (Pfaller *et al*., 2007). Limited treatment options and the high adaptive capability

of fungal pathogens to stress conditions associated with colonization of different host niches and drug exposure (Kołaczkowska and Kołaczkowski, 2016) further increments the importance of these infections.

1.3.1 Most common fungal pathogens in humans

Recent epidemiologic data from various studies show that invasive fungal infections are frequently encountered in clinical practice, with *Candida* spp. and *Aspergillus* spp. as the most common species (Arvanitis *et al*., 2014). However, other previously less common and emerging fungal pathogens are being increasingly diagnosed in patients at risk of invasive fungal infections (Klepser, 2011).

Candida spp. are present in the oral cavity, digestive tract and genital region as the commensal flora in more than half of the healthy population (Cui *et al*., 2015). However, under conditions that weaken host defense mechanisms (e.g. patients who are severely immunocompromised, have endured invasive clinical procedures, or have experienced major trauma, requiring extended stays in intensive care units (Brown *et al*., 2012)) they may become opportunistic pathogens, causing localized mucosal disease or life-threatening invasive infections. These infections have an unusual high mortality rate despite antifungal therapy (Lionakis and Netea, 2013). In these cases, *Candida* spp. may cause invasive disease, most often as bloodstream infection (candidaemia), with or without secondary dissemination to the eyes, liver, spleen, bones, heart valves, central nervous system, or as deep-seated candidiasis, such as peritonitis after gastrointestinal surgery (Arendrup, 2010). Candidaemia alone is responsible for 10% of nosocomial diseases in ICUs (Bansal and Pande, 2013) and candidiasis, the fourth leading cause of nosocomial bloodstream infection in the United States, has an estimated annual cost of US$2 billion (Lionakis and Netea, 2013), making *Candida* species as medically important as many mainstream bacterial infections (Moyes and Naglik, 2011). Candidemia and other forms of invasive candidiases are unquestionably the most prevalent of the invasive mycoses worldwide (Pfaller *et al*., 2014).

More than a dozen *Candida* species can cause disease, but in almost all patient groups and disease manifestations, *C. albicans* dominates in terms of incidence (Pfaller *et al*., 2007, 2014; Arendrup *et al*., 2013; Bansal and Pande, 2013; Delarze and Sanglard, 2015; Deodato *et al*.,

2016), being globally responsible for 50 to 70% of all cases (Arendrup, 2010). However, other emerging *Candida* species including *C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis* are now posing serious nosocomial threats to the patient population (Chakrabarti *et al*., 2009). Differences in epidemiology are observed comparing various geographical regions, age groups and patient groups, and changes in rate and species distribution and susceptibility have been observed over the recent decades (Arendrup, 2010). For example, *C. glabrata* is more frequent in the older population than in middle-aged adults (Calandra *et al*., 2016) and is more common in the northern hemisphere, while *C. parapsilosis* is more often encountered in the southern parts of the world and in Asia (Kullberg and Arendrup, 2015). The changing epidemiology has also been partly attributed to the selection of less sensitive *Candida* strains by the widespread use of the azole fluconazole as a prophylactic and therapeutic agent (Sanguinetti *et al*., 2015). Given the differential intrinsic susceptibility patterns exhibited by different *Candida* species, the primary antifungal regimes should be adjusted to the local epidemiology (Arendrup *et al*., 2013).

Among molds, *Aspergillus fumigatus* is the most frequent specie of *Aspergillus* causing clinical disease, perhaps due to specific virulence factors unique to this organism (Person *et al*., 2010). *A. fumigatus* is a ubiquitous saprophytic fungus to which humans are exposed daily in most parts of the world. The infection is initiated by inhalation of conidia, which are readily destroyed in a normal host but can cause invasive disease in immunocompromised individuals (Kwon-chung and Sugui, 2013).

A. fumigatus most commonly causes invasive pulmonary aspergillosis, often followed by dissemination throughout the body (Person *et al*., 2010), but almost any organ can be infected, with disseminated aspergillosis defined as active infection in two or more non-contiguous organs (Dimopoulos *et al*., 2003). Invasive aspergillosis is the second most common invasive fungal infection, with increasing incidence over the last 20 years along with the advances in the treatment of hematological malignancies (Oren and Paul, 2014). Despite improvements in diagnostic modalities and the antifungal armamentarium, including broad-spectrum azoles and the echinocandin antifungals, mortality remains high (Baddley *et al*., 2013), with the incidence of invasive aspergillosis among ICU patients being estimated to be as high as 7% (Baddley *et al*., 2013). Left untreated, mortality rates from invasive pulmonary aspergillosis exceed 90% and even following aggressive antifungal treatment, fatality rates of 50% are common in heavily

immunosuppressed hosts such as those with leukemia or transplant recipients (Osherov and Kontoyiannis, 2016). This attributable mortality associated with disseminated aspergillosis is partly due to difficulties in diagnosing the disease during its early stages. Indeed, diagnosis of disseminated or invasive aspergillosis is often made only at autopsy (Dimopoulos *et al*., 2003).

Even though *A. fumigatus* is the most common human mold pathogen causing severe and frequently fatal infection in patients with immunosuppression or underlying structural lung disease (Osherov and Kontoyiannis, 2016), other species are emerging, most commonly *A. flavus, A. terreus*, and *A. niger* (Person *et al*., 2010).

1.4 The fungicides and the antifungal pipeline

1.4.1 Plant protection products (PPP)

Fungicides have been used for over 200 years to protect plants from fungal pathogens. From localized applications and rudimentary and primitive fungicides, mainly used to protect cereal seeds and grapevines, the number of crops and crop diseases treated, the range of chemicals available, the area and frequency of administration and the effectiveness of treatments have increased enormously, especially since the second world war (Brent and Hollomon, 1995).

For plant disease control, chemicals are a critical element in effective integrated pest management programs. By the early 19th century, sulfur and copper compounds were already in use, both as foliar sprays and seed treatments, to control a number of diseases (Hollomon, 2015). Fungicides that have multiple mechanisms of action with preventive and curative properties against several target sites in fungal metabolism played a leading role in the first half of the 1900s, while fungicides inhibiting a specific target site were introduced in the 1960s (Hirooka and Ishii, 2013). Some fungicides are active towards a wide range of fungal diseases, whereas others have a limited spectrum of activity against one or two specific groups of plant pathogens (Brent and Hollomon, 1995). World sales of fungicides for crop use totaled US$9.91 billion in 2010 and have increased 6.5 % annually since 1999 (Hirooka and Ishii, 2013).

In contrast to antifungal drugs targeting human diseases, agricultural fungicides are much more diverse in their biochemical modes of action and chemical structures (Myung and Klittich, 2015; Jampilek, 2016). This was expected since a much higher toxicity level is tolerated in fungicides

applied in agriculture than in medicine. Currently, at least forty-five different modes of action (www.FRAC.info 2015) are identified for agricultural fungicides (Hollomon, 2015). The different chemical groups of agricultural fungicides include sterol biosynthesis inhibitors (SBIs), strobilurins, pyrazole carboxamides, morpholines, benzimidazoles, anilinopyrimidines, henylpyrroles and several others (Myung and Klittich, 2015). The international Fungicide Resistance Action Committee (FRAC) has grouped agricultural fungicides according to their mode of action, with more than ten different biochemical processes as their target sites (Hirooka and Ishii, 2013; Myung and Klittich, 2015). Examples are the sterol biosynthesis inhibitors (DMIs), quinone outside inhibitors (QoI), succinate dehydrogenase inhibitors (SDHIs), and inhibitors of β-tubulin assembly during mitosis (Benzimidazoles), among others (Brent and Hollomon, 1995; Knight *et al*., 1997;Hirooka and Ishii, 2013).

1.4.2 Antifungal drugs to treat human infections

Currently, 12 drugs from four antifungal drug classes are approved for the treatment of systemic fungal infections in the USA (Andes, 2013; Delarze and Sanglard, 2015). These include four different formulations of polyene amphotericin B (amphotericin B deoxycholate, amphotericin B lipid complex, amphotericin B colloidal dispersion and liposomal amphotericin B), four drugs of the azole class (fluconazole, itraconazole, voriconazole and posaconazole), three drugs from the echinocandin class (caspofungin, micafungin and anidulafungin) and one pyrimidine (flucytosine, also known as 5-fluorocytosine or 5-FU) (Myung and Klittich, 2015). Each antifungal compound has advantages and limitations related to its spectrum of activity, route of administration, drug interactions and toxicity profile. Only a very small number of targets are involved, namely ergosterol and its biosynthesis, nucleic acid synthesis, and cell wall synthesis (Cowen *et al*., 2002).

Polyenes are produced by the bacterial genus *Streptomyces* and were first reported in 1955 (Denning and Hope, 2010). The polyene class of antifungal drugs can now be divided into 2 subclasses: conventional polyenes (amphotericin B-deoxycholate) and lipid-based formulations of amphotericin B (Klepser, 2011). Compared with amphotericin B-deoxycholate, lipid formulations of amphotericin B are better tolerated, with improved side-effect profiles and reduced nephrotoxicity (Nett and Andes, 2016), allowing higher doses to be administered (Klepser, 2011). Despite extensive investigation for the last 50 years, the mechanism(s) of action of amphotericin B remains unclear (Gray *et al*., 2012). The classical action mechanism

involves amphotericin B binding to cell membrane ergosterol, with subsequent pore formation and ion leakage. However, different findings suggest that this may not be the main action of this molecule. Different chemical modifications of the molecule were studied and used to show that when the molecular changes resulted in the inability to form pores, amphotericin B still retained antifungal activity (Palacios *et al*., 2007). It has also been shown that amphotericin B induces the intracellular accumulation of reactive oxygen species (ROS) (Mesa-Arango *et al*., 2014). Furthermore, a genome-wide expression analysis confirmed that amphotericin B not only affects the expression of genes involved in ergosterol synthesis but also promotes the expression of stress genes (Liu *et al*., 2005). Despite the development of novel antifungal drugs in the last two decades, amphotericin B has been considered the "gold standard" among antifungal drugs and remains the active ingredient with the broadest antifungal spectrum (Hamill, 2013), presenting fungicidal activity against both yeasts and molds (Klepser, 2011). Although the structural difference between ergosterol and cholesterol (the major sterols in fungal and mammalian membranes, respectively) is sufficient to explain the greater binding affinity of amphotericin B to ergosterol over cholesterol, this selectivity is low and suggests a potential toxicity for mammalian cells (Odds *et al*., 2003). In fact, its clinical use is hampered by a high incidence of infusion-related adverse events and a substantial incidence of renal toxicity (Hamill, 2013; Nett and Andes, 2016). Over 1.5 million people die from invasive fungal infections each year, largely because the extreme toxicity of amphotericin B is dose-limiting (Davis *et al*., 2015).

In the 1990s a new class of antifungal agents, the azoles, were introduced in the market. Azoles are cyclic organic molecules which can be divided into two groups on the basis of the number of nitrogen atoms in the azole ring: the imidazoles contain two nitrogen atoms, and the triazoles contain three nitrogen atoms (Maertens, 2004). While the original imidazole drugs (ketoconazole, miconazole) exhibit severe toxicity during systemic administration, the newer triazoles (fluconazole, itraconazole, posaconazole, voriconazole, isavuconazole) have an improved safety panel (Nett and Andes, 2016). Azoles are a fungistatic drug class that inhibit growth, but do not kill the fungus, thereby providing the opportunity for the development of resistance mechanisms (Morschhäuser, 2016). Azoles disrupt the cell membrane by inhibiting the activity of lanosterol 14α-demethylase, an enzyme coded by the *ERG11* gene (Mishra *et al*., 2007) and involved in the biosynthesis of ergosterol (Spampinato and Leonardi, 2013). Its activity replaces ergosterol with unusual sterols and the normal permeability and fluidity of the

resulting fungal membrane is impaired (Odds *et al*., 2003). Therapies for systemic candidiasis commonly treated with fluconazole and amphotericin B may be unsuccessful. This situation is further aggravated by the emergence of drug-resistant *Candida* strains of both *albicans* and non-*albicans* species to fluconazole. These observations represent an increasingly worrisome challenge to clinicians (Klepser, 2006; Ahmad *et al*., 2013).

The echinocandins, a novel class of antifungal compounds now widespread in clinical use, are semisynthetic derivatives of fungal-derived cyclic hexapeptides (Denning and Hope, 2010) and were introduced in 2001 with caspofungin (Nett and Andes, 2016). Echinocandins inhibit the synthesis of β-(1,3)-D-glucan synthase, by targeting its subunits, which are encoded by the *FKS1, FKS2* and *FKS3* genes (Kartsonis *et al*., 2003). This results in cell wall disruption, as the product of this enzyme, β-(1,3)-glucan, is an essential component for the fungal cell wall infrastructure (Bansal and Pande, 2013). Echinocandins exhibit low toxicity, since they have only one target enzyme that is not present in mammalian cells (Klepser, 2011). They also display a broader spectrum of activity, lower drug interaction, few resistance problems and higher efficacy against *Candida* spp. (Bansal and Pande, 2013). Their favorable safety profile and broad-spectrum anti-*Candida* spp. activity make them the first-line therapy for invasive candidiasis (Alexander *et al*., 2013). However, there are already reports of resistant strains against echinocandins (Bansal and Pande, 2013), especially among fluconazole-resistant isolates (Alexander *et al*., 2013), a probable consequence of their misuse.

The antifungal drug 5-flucytosin, a pyrimidine analogue, enters fungal cells through one or more permeases and is then converted, by cytosine deaminase, to its metabolically active form 5-fluorouracil (5-FU) (Costa *et al*., 2015). 5-FU gets incorporated into DNA and RNA during their syntheses (Prasad *et al*., 2016), inhibiting DNA replication, transcription and protein synthesis (Costa *et al*., 2015). Most filamentous fungi lack the enzyme(s) involved in this process and hence the useful spectrum of flucytosine is restricted to pathogenic yeasts (Odds *et al*., 2003). Although flucytosine exhibits activity against most *Candida* spp, its use as a single agent in antifungal treatment is quite limited because it rapidly promotes resistance (Nett and Andes, 2016). Its application is most valuable as adjunctive treatment with amphotericin B and fluconazole in clinically difficult infections (Espinel-Ingroff, 1998; Odds *et al*., 2003). The use of two or more antifungal drugs to control severe invasive fungal infections has been adopted for a long time in the treatment of invasive candidiasis, and it is known as synergistic therapy (Cui

et al., 2015). Synergistic drug combination has been proved to be a valid and pragmatic strategy to seek drugs with novel mode of actions. It can potentially reduce the dose of single drug usage with increased drug-efficacy, and subsequently lower the drug toxicity (Cui *et al*., 2015), while decreasing drastically the chances of resistance development.

Unlike antibiotics, the repertoire of antifungals is rather limited (Ngo *et al*., 2016) and the antifungal drug discovery pipeline has declined substantially over the past decades (Cui *et al*., 2015). One of the reasons for such decline is undoubtedly the small market size as compared to antibiotics (Myung and Klittich, 2015). One other important motive is the fact that many potential antifungal drug targets have homologs of similar function and susceptibility for inhibition in humans, and therefore, their toxic side effects dramatically reduce the number of antifungal agents that can be used therapeutically (Cowen *et al*., 2002), limiting the scope of drug discovery and development (Brown *et al*., 2012). In fact, countless highly potent antifungal molecules have been discovered over the last decades but only a few of them have become viable therapeutic candidates (Lewis, 2011).

1.5 The rising threat of fungal resistance

Over the last 35 years agriculture has faced problems arising from the development of resistance in fungal pathogens of crops, against the fungicides used to control them (Brent and Hollomon, 1995). The use of synthetic fertilizers and pesticides has become an integral part of modern agriculture but their use suffers from increasing problems of resistance in the target organisms (Hahn, 2014). Moreover, pathogen populations that develop resistance to one fungicide become automatically and simultaneously resistant to those other fungicides which are affected by the same gene mutation and the same resistance mechanism (Brent and Hollomon, 1995). By far, the commonest resistant mechanism appears to be an alteration to the biochemical target site of the fungicide. This could explain why many of the older active ingredients have not encountered resistance problems. Once they have penetrated the fungal cell, the older fungicides act as general enzyme inhibitors, affecting many target sites (hence they are sometimes called 'multi-site' inhibitors). They act selectively on fungi, rather than on plants and animals, because they tend to penetrate and accumulate much more readily in fungi. Many target sites in the fungus would have to change simultaneously in order to stop the fungicide from working (Brent and Hollomon, 1995).

Fungicide resistance was first reported in the field in 1971, when the efficacy of two antibiotics, polyoxin and kasugamycin, decreased against black spot disease on Japanese pear (pathogen: *Alternaria alternata* Japanese pear pathotype) and blast disease on rice (*M. oryzae*), respectively. Since then, fungicide resistance has not stopped to cause problems, repeatedly decreasing fungicide efficacy on various crops (Hirooka and Ishii, 2013). Up to 1970 there were a few sporadic cases of fungicide resistance, which occurred many years after the fungicide concerned was introduced. With the development of systemic fungicides, the incidence of resistance increased greatly, and the time taken for resistance to emerge became often relatively short, sometimes within two years after market entrance (Brent and Hollomon, 1995). According to Brent and Hollomon (Brent and Hollomon, 1998), the groups with the higher resistance-development risk are the phenylamides and strobirulins, whereas antifungal drugs with a multi-site mode of action are less likely to induce resistance development (Hollomon, 2015).

With regard to infectious diseases of humans, it is likely that agricultural application of fungicides has also led to the development of resistance in fungal pathogens that cause diseases in the clinical area. Recent studies reported the emergence and spread of multi-resistant strains of *A. fumigatus* resulting from the agricultural use of triazole fungicides (Chowdhary *et al*., 2013; Rivero-menendez *et al*., 2016; Verweij *et al*., 2016).

In general, antifungal drug resistance can be divided in microbiological or clinical. Microbiological resistance is defined as the non-susceptibility of a fungal pathogen to an antifungal agent as determined by *in vitro* susceptibility testing and compared with other isolates of the same species (Sanguinetti *et al*., 2015). In contrast, clinical resistance refers to the persistence of a fungal infection after treatment with an adequate therapy. This type of resistance may result from problems in drug pharmacokinetics, drug–drug interactions or patient immunity (Cowen *et al*., 2002). Microbiological resistance can be further characterized as natural or acquired. In natural resistance, the microorganisms lack the target structure of the drug, and all isolates of the species are resistant, even though they have had no previous exposure to the agent (Klepser, 2006). In acquired resistance, the microorganisms develop the ability to resist to the drug to which they were previously susceptible (Jampilek, 2016). Acquired resistance is most common in isolates exposed to relatively low concentrations of the drug for prolonged periods - for example, in patients infected with human immunodeficiency

virus (HIV) who receive multiple courses of antifungal therapy for recurrent oropharyngeal candidiasis (Klepser, 2006). Acquired resistance evolves because antimicrobial agents are rarely deployed in a way that completely eradicates the pathogen population in the host, and the survival pathogen cells are then subjected to natural selection. Whenever the pathogen population remains large enough over the course of drug treatment, the evolution of resistance is inevitable (Cowen *et al*., 2002). For this reason, the acquired resistance is considered the most serious case of resistance (Jampilek, 2016).

Acquired resistance to antifungals develops by mutations on specific genes (Cowen *et al*., 2002; Sanglard and Odds, 2002) and by the ability of the microorganisms to adapt to changes in their environment by appropriately adjusting gene expression and cellular activities (Morschhäuser, 2016). Mechanisms of resistance to antifungals differ among groups of drugs mainly due to the mode of action of each class of antifungals (Jampilek, 2016), and can be divided in three main groups: (i) reducing drug intracellular accumulation, (ii) decreasing the affinity of the drug for its target, and (iii) counteraction of the drug effect (Mishra *et al*., 2007; Vandeputte *et al*., 2012; Spampinato and Leonardi, 2013; Ngo *et al*., 2016; Sanglard, 2016). Reports on novel resistance profiles have appeared recently, including the development of multi-drug resistances (Sanglard, 2016).

Four main mechanisms of azole resistance have been reported among *Candida* species (Kanafani and Perfect, 2008): loss of function of the *ERG3* gene that codes for a Δ5,6-desaturase, thus preventing the accumulation of toxic sterol (Morschhäuser, 2016); alteration or up-regulation of the target enzyme codified by *ERG11* gene, decreasing its affinity for the fungicidal inhibitor (Kołaczkowska and Kołaczkowski, 2016) or leading to a situation where the drug is overwhelmed by an anomalously large amount of the target enzyme, respectively (Sanguinetti *et al*., 2015); and overexpression of the transcription factors *MRR1* and *TAC1*, that control the expression of *MDR1* and *CDR1/CDR2*, respectively, two multi-drug pumps that transport fluconazole out of the cell (Grossman *et al*., 2015; Morschhäuser, 2016).Resistance to echinocandin drugs among clinical isolates is associated with amino acid substitutions in two "hot-spot" regions of *FKS1*, a gene that codes for the major subunit of glucan synthase complex, the presumed target of these fungicides (Perlin, 2007; Kanafani and Perfect, 2008). Regarding resistance to flucytosine, some yeast strains are intrinsically resistant because they have a mutation in cytosine permease, encoded by the *FCY2* gene, the enzyme responsible for its entry

in the cell. In these strains, cellular uptake of flucytosine is therefore inhibited. Acquired resistance is also a problem and results from defects in flucytosine metabolism through mutations in cytosine deaminase or uracil phosphoribosyl transferase (encoded by *FCY1* and *FUR1* genes, respectively) (Papon *et al*., 2007; Kanafani and Perfect, 2008; Costa *et al*., 2015). Despite more than 30 years of clinical use, minimal resistance to amphotericin B has been developed (Kanafani and Perfect, 2008; Davis *et al*., 2015). However, the main problem associated with the prophylactic use of conventional amphotericin B has always been due to its well-known side effects and toxicity (Spampinato and Leonardi, 2013; Delarze and Sanglard, 2015; Nett and Andes, 2016). Whereas drugs with multiple targets are usually associated with higher toxicity, drugs that strongly inhibit a single step in a pathway are more likely to promote selective pressure and resistance development (Wong *et al*., 2014).

Resistance to antifungal drugs poses a challenge in the treatment of invasive fungal infections. Some fungal pathogens display innate resistance or reduced susceptibility to drugs, and treatment with antifungal agents can itself lead to acquired resistance (Klepser, 2011). For example, *Candida* species exhibit variable degrees of susceptibility to the most commonly used antifungal agents (Sanguinetti *et al*., 2015): *C. glabrata* and *C. krusei* are the most frequent species with reduced susceptibility to one or several azoles, *C. parapsilosis* is the most common species with decreased susceptibility to echinocandins and *C. lusitaniae* is the less susceptible to amphotericin B (Arendrup, 2010).

Antifungal drug resistance is a matter of great concern because there is a limited number of drug classes targeting different fungal components and because the number of patients at risk receiving treatment is continually growing, thus further increasing the use (and misuse) of antifungal drugs (Maubon *et al*., 2014). Furthermore, there is currently an alarmingly high proportion of EPA-approved molecules used in crops that are either structurally or pharmacologically the same as the FDA-approved drugs utilized in medicine, something which has been demonstrated to contribute to widespread fungal resistance (Ngo *et al*., 2016).
Comparing to antibiotic resistance, antifungal resistance has received much less attention, although the occurrence of fungal diseases is far from negligible (Delarze and Sanglard, 2015). There is a demand for novel compounds, with new modes of action, to treat these resistant forms (Butts and Krysan, 2012; Bansal and Pande, 2013; Jampilek, 2016) that emerged after the use (and misuse) of antifungal agents launched more than 20 years ago (Deodato *et al*.,

2016). Moreover, the majority of antifungal drugs usually target essential pathways which cause the evolution of drug resistance rapidly, such as azoles and echinicandins, while resistance to amphotericin B is rare (Cui *et al*., 2015). The available evidence suggests that development of drug resistance can be effectively slowed down by the multi-target strategy (Cui *et al*., 2015).

1.6 The need for a next generation of 'smart' fungicides

In crop production, 30 to 70% of the yield can be lost to disease in the absence of fungicides. Even with the current usage rate of fungicides, 16% of the theoretical yield is still being lost (Oerke, 2006). However, the environmental and societal damages attributed to the use of fungicides are estimated at US$9.6 billion due to their negative impact on public health, livestock product losses, destruction of natural enemies and development of pesticide resistance, crop pollination problems, honeybee, crop and wildlife losses, and governmental expenditures to reduce the environmental and social impact (Peshin and Zhang, 2014). In this scenario, policy decision makers will have to make balanced decisions that, at the same time, insure social and environmental sustainability, and allow for the use of safe fungicides that increase the crop yields required to feed the world population. The focus of the EU Research and Innovation program (Europe 2020 Strategy) (Europe 2020), embraces the use of renewable resources and priories the consideration on the degradation of the environment that may cause serious damage to human health while destroying the ecosystem. Amid these concerns, the restrictions to the use of fungicides are motivated by environmental impacts and human toxicity, global warming and depletion of the ozone layer, and call for the urgent development of new products to bypass the cycle imposed by the use of chemical formulations.

Evidence collated on a worldwide basis since 1950 indicates a pattern of decline in the number of new active substance introductions since 1997 (McDougal, 2013). Of greater concern, however, is the fact that there has been a significant fall in the number of new active substances in development from 70 in 2000, to just 28 in 2012. The share of investment in global crop protection for products used in Europe has fallen from 33.3% in the 1980s to only 7.7% in the 2005-2014 period (McDougal, 2013). Whilst Europe was benefitting from new agrochemical introductions at a rate of 4.1 and 4.0 products per annum in the 1980s and 1990s, respectively, this rate has been predicted to fall significantly to 1.2 per annum between 2005 and 2015

(McDougal, 2013). There are a number of inter-related factors which have caused the decline in R&D investment in Europe, the most important of which are the costs associated to the difficult approval process. The most recent data on the average cost of bringing a new active substance to market indicates that there has been a significant increase between 1995 and 2005 of 68.4% (McDougal, 2010). Of particular note is that whilst research costs have risen by 18.0%, a staggering 117.9% of the rise relates to development costs (studies required to achieve registration). This large increment in time-to-market and costs is mostly due to the huge amount of data that must be supplied in terms of studies to support an application. The current high risks of failing approval somewhere along the certification process further contributes to the observed decline in the number of new fungicides entering the market. This is a direct result of a more risk-averse regulatory environment. Some of the cost is for very little gain in terms of safety. This is likely to create a large amount of research work through comparative studies, but with minimal or no improvement in safety or environmental outcomes. In many cases, the modelling work and risk assessments are not representative of real-world situations in which the products are to be used. Paradoxically, the improvement in science has contributed to the mushrooming cost of approvals. Better scientific knowledge and equipment creates more and better data. It provides more issues to look at and causes concerns to be raised. All these problems apply not just to the approval and registration of new active substances. They also apply to the re-registration of existing pesticides. In a lot of cases, the investment spent on securing a re-registration is many times the original cost of getting the pesticide approved.

The overall situation in what human fungal infections are concerned is not any better. A big hurdle in the treatment of invasive mycoses is the serious shortcomings of diagnostic methods or their late deployment, very often leading to deaths that could have been avoided. Most diagnostic kits lack sensitivity, speed and specificity, and many are unaffordable or inappropriate for the needs of developing countries, where invasive fungal infections are much more serious and widespread. The second impediment is the relative small arsenal of commercially available antifungal drugs. Those that are available have so far only managed to make a small dent in the high mortality rate of patients, because of the lack of early diagnosis, drug toxicity and interactions, as well as limitations in spectrum of action and mode of administration. More worryingly, certain fungal pathogens are becoming resistant to the current existent antifungal drugs (Agarwal-Jans, 2016).

Although there is a pressing need to develop new drugs that inhibit novel fungal-specific targets, no new classes of antifungals have been commercialized since the launch of the echinocandins in 2001. Most of the large pharmaceutical companies have downsized or even halted their antifungal discovery efforts. Development of treatments for much more frequent and chronic diseases (e.g. diabetes mellitus) is far more lucrative. Discovering new antifungals when screening established compound libraries or natural products has been proven difficult for a variety of reasons, chiefly because fungi are eukaryotes, with a metabolism closer to ours than to that of bacteria (Mayr and Lass-Florl, 2011). In the 1990s, following the introduction of new tools in genomics and molecular biology, the large pharmaceutical companies did perform expensive large-scale antifungal drug screening projects (Perfect, 2016). However, these efforts led to the rediscovery of known compounds (and in particular azoles and glucan-synthase inhibitors, known as "the low-hanging" fruit) or "hits" with unacceptable toxicity in mammals (Roemer *et al*., 2011; Roemer and Krysan, 2014). The playing field has been largely left to the efforts of small biotech startups and individual research labs, which lack the tremendous resources needed to push a drug through human trials and the extensive regulatory oversight associated with phase I–III drug development.

In an attempt to address the lack of investment for the development of new antifungals, the U.S. FDA Generating Antibiotic Incentives Now (Gain) Act, allows a 5-year extension of market exclusivity for new antifungal drugs intended to treat serious or life-threatening infections (Denning and Bromley, 2015). The lack of new antifungal drugs and the limited therapeutic options call for urgent strategies to find novel antifungal candidates (Cui *et al*., 2015).

1.7 Novel, non-conentional strategies to control fungal infections

1.7.1 Biopesticides

Biopesticides are plant protection products (PPP) that are naturally occurring substances that control pests by nontoxic mechanisms. Biopesticides are living organisms (natural enemies) or their products (phytochemicals, microbial products) or byproducts (semiochemicals) which can be used for the management of pests that are injurious to plants. Three main types are available in the EU. Microbial pesticides comprise a living microorganism (e.g. a bacterium, fungus, virus or protozoan) as the active substance. Examples of microbial pesticides are *Bacillus thuringiensis*, a bacteria used to control *B. cinerea* in fruit, and the fungus *Coniothyrium*

mintans, to control sclerotinia in oilseed rape Biochemical pesticides are naturally occurring substances that have plant protection properties. Examples are plant derived products or insect pheromones. Finally, there are Plant-Incorporated Protectants consisting of pesticidal substances that plants produce from genetic material that has been added to the plant (Sarwar, 2015). These products display a number of advantages. Unlike 'synthetic' pesticides, biopesticides are usually fully bio-degradable and to not leave contaminating metabolites. There is usually no withdrawal period after the use of biopesticides. Their mode of action is often very restricted – only attacking a specific target organism. This means they do not affect other parts/elements of the environment.

A number of drawbacks have been suggested for biopesticides. Perhaps the most worrying risk of using microbial fungicides concerns the environment microbiomes. The biological importance of the microbiomes which naturally colonize all biological environments has only recently started to be appreciated. The well known observation that only ca 1% of the microorganisms naturally present in a biological sample grow under *in vitro* conditions has hampered microbiome analyses. However, with the development of high throughput metagenomic tools it is now possible to detail the composition of most microbiomes. The application and release of zillions of viable microbial cells may affect the environment in unpredictable ways, possibly by inevitably disrupting natural microbiomes, something that may pass unnoticed for a considerable amount of time. In addition, biopesticides are perceived to be more costly than synthetic PPP (although this is not necessarily the case). The products tend to be slower acting, which means that they are more suited to preventative applications, than immediate treatment of an outbreak. A sufficient density of the control product must be maintained over a period of time for the biopesticide to take effect. Sometimes this can be problematical due to all the varying climatic and biological factors in play under field situations. Multiple applications may be required rather than one or two applications of synthetic PPPs. In general, it might be considered that the use of these products requires a 'mindset' change (Chandler *et al*., 2011). They are more about controlling fungal populations below levels that are injurious to the crop, rather than their complete removal. There is a greater degree of management required than with conventional pesticides. This is due to the need for multiple applications, and the requirement to use the product before each fungus becomes established. This partly explains their greater take-up in the specialist cropping sector, where the areas of crop are smaller, the values higher and the management more intensive.

1.7.2 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) constitute a primitive mechanism of innate immunity and form the first line of defense to protect hosts from their microbial attackers (Selitrennikoff, 2001; Hegedüs and Marx, 2013). AMPs have a widespread distribution throughout the animal and plant kingdoms, suggesting they have played a fundamental role in the successful evolution of complex multicellular organisms (Zasloff, 2002). They are typically derived from larger precursors, often suffering several post-translational modifications (Zasloff, 2002). More recently, it became evident that these peptides exhibit a wide range of biological activities, from direct killing of invading microbes to modulation of innate immune response and other biological host defenses (Yeung *et al*., 2011).

AMPs are a unique and diverse group of molecules, which are divided into subgroups on the basis of their amino acid residue sequence and structure (Brogden, 2005). Their antifungal activity and consequent therapeutic potential have been recognized since the 1990s. Although the bulk of efforts on commercializing AMPs has remained focused on their potential antibacterial applications, bioactivity against a range of fungal pathogens relevant to human health has been confirmed for a number of human, mammalian, amphibian, insect and plant derived AMPs (Duncan and O'Neil, 2013). Because of their potent antifungal activity, there has been a great deal of interest in exploiting these peptides as commercial antifungal agents in agriculture as well as in medicine (Shah and Read, 2013). In fact, significant research has been carried out to develop these naturally occurring antifungal peptides as therapeutic agents against microbial infections (Zasloff, 2002; Brogden, 2005; Fjell *et al*., 2012; Virágh *et al*., 2015; Garrigues *et al*., 2016).

A main hurdle that has hindered the development of antimicrobial and antifungal proteins as therapeutic agents is the fact that many naturally occurring proteins exhibiting antifungal activity *in vitro* (e.g. magainin) are only effective *in vivo* at very high doses, often close to the toxic doses of the peptide (Darveau *et al*., 1991; Zasloff, 2002). The literature is full of reports describing failed attempts to introduce new antimicrobial peptides/proteins in the market (e. g. polyphemusin, a peptide that inhibits the growth of various Gram-positive and Gram-negative bacteria as well as *C. albicans*, but exhibits haemolytic activity; a protegrin derivative that failed in phase III clinical trials of oral mucositis (Fjell *et al*., 2012)). Only four antimicrobial peptides, effective especially against *Candida* spp. and *Aspergillus* spp., are now in the second

phase of clinical trials: $(CKPV)_2$ peptide (CZEN-002) for the treatment of candidiasis and vaginitis; human lactoferrin 1–11 (hLF1–11) for the treatment of infectious complications among haematopoietic stem cell transplant recipients (van der Velden *et al*., 2009); PAC113 (P-113) for the treatment of oral candidiasis in HIV seropositive patients; and NP339/NP525 for the treatment of fungal disease as an inhaled, systemic and mucocutaneous therapy (Jampilek, 2016).

Another historic hurdle for the commercialization of peptide therapeutics for systemic application, has been their biological stability. In comparison to their smaller molecular counterparts, peptides are more susceptible to proteolytic degradation in the systemic environment resulting in shorter half-lives and making it more difficult to maintain the plasma concentrations needed for their minimal inhibitory concentration (Duncan and O'Neil, 2013). Bioactive proteins are even more unstable, due to the risks of unfolding and denaturation. It should be noted that many of these bioactive proteins/peptides are part of the host defensive mechanisms, meaning that their stability is usually far greater than those of housekeeping oligomers/polymers.

Due to the vast variety in function, structure, expression pattern, target organisms and producing hosts, the classification of AMPs is difficult and somewhat arbitrary up to date. Mostly, AMPs are usually classified according to their functional and/or structural properties. The most prominent group within the antifungal proteins are the defensins from plants, insects and mammals (Hegedüs and Marx, 2013). Defensins are a family of evolutionary related antimicrobial peptides with a characteristic β-sheet-rich fold and framework of six disulfide-linked cysteines (Ganz, 2003). They are particularly abundant in seeds but have also been described in leaves, pods, tubers, fruit, and floral tissues (Van Der Weerden *et al*., 2008). Because of their potent antifungal activity, defensins have been widely exploited in agro biotechnological applications to generate disease resistant crops (Sagaram *et al*., 2013). Antifungal plant defensins are quite diverse in their modes of action, and several mechanisms have already been described. For example, Psd1 from *Pisum sativum* acts at the cell cycle level, blocking the roles of cyclin F in the S to G2 phase transition (Lobo *et al*., 2007). Nad1 from *Nicotiana alata* (Lay *et al*., 2003) binds to the cell wall, and rapidly permeabilizes the fungal plasma membrane, allowing its entry into hyphal cells. Whether death then occurs due to membrane leakage or to the interaction of NaD1 with intracellular targets, which induces a

programmed cell death, is still unknown; however, the production of ROS does suggest an apoptosis-like phenotype (Van Der Weerden *et al*., 2008). MtDef4, from *Medicago truncatula*, rapidly binds to phosphatidic acid, permeabilizes fungal plasma membrane and is internalized by the fungal cells where it accumulates in the cytoplasm, affecting several intracellular targets (Sagaram *et al*., 2013).

Membrane permeabilization is a common activity for many antimicrobial peptides, although the mechanism of permeabilization can differ significantly, and in some cases, it remains unclear (Van Der Weerden *et al*., 2008). However, *in vitro* studies have revealed that direct antimicrobial activity is not limited to the previously suggested mechanisms of membrane and/or cell rupture but it actually extends to the membrane-associated biosynthesis, to the macromolecular synthesis in the cytoplasm and to several metabolic functions (Brogden, 2005; Yeung *et al*., 2011; Vriens *et al*., 2016). Either way, it is now clear that some antifungal peptides exert their antifungal action just by binding to specific components of the fungal plasma membrane, whereas other peptides are internalized by fungal cells and have intracellular targets (Shah and Read, 2013).The precise mechanisms of the antifungal action of defensins that are capable of entering into the fungal cells are still not fully understood and the sequence motifs mediating fungal cell entry of these defensins remain to be identified (Sagaram *et al*., 2013). Nevertheless, unlike conventional antimicrobial agents, which microbes readily circumvent, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is quite improbable because the main target of antimicrobial peptides is the plasma membrane. A microbe would have to redesign its membrane, changing the composition and/or organization of its lipids, which is probably a 'costly' solution for most microbial species (Zasloff, 2002).

1.7.3 Recombinant proteins as a source of new antifungal molecules

The great majority of biopharmaceuticals that have been approved for therapeutic applications by regulatory authorities are proteins that have been produced by means of recombinant DNA technology in various expression systems (Berlec and Strukelj, 2013). *Escherichia coli*, yeast and mammalian cells together account for the production of 89% of approved biopharmaceuticals. The other 11% of approved biopharmaceuticals are produced by hybridoma cells (some monoclonal antibodies), insect cells, transgenic animals, plants, or other

hosts (Berlec and Strukelj, 2013). *E. coli* remains one of the most attractive expression systems because of its ability to grow rapidly and at high density on inexpensive substrates, its well characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Baneyx, 1999; Baneyx and Mujacic, 2004; Dyson *et al*., 2004; Huang *et al*., 2012; Khow and Suntrarachun, 2012). The major drawbacks of *E. coli* as an expression system include the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of proteins into the culture medium, and the limited ability to facilitate extensive disulfide bond formation (Makrides, 1996; Huang *et al*., 2012).

The production of proteins, whether for biochemical analysis, therapeutics or structural studies, requires the successful achievement of three individual goals: expression, solubility and purification (Esposito and Chatterjee, 2006). Typically, recombinant proteins are expressed in *E. coli* with small affinity tags, such as hexahistidine (His-tag) to allow the subsequent efficient isolation of the desired protein by affinity chromatography (Hewitt *et al*., 2011). His-tag, which binds to immobilized transition metals, is by far the most commonly used affinity tag for high-throughput protein purification. His-tag combines the advantages of small size with the added benefit of interacting with a chromatographic matrix (e.g. Ni-NTA resin) that is relatively inexpensive, able to withstand multiple regeneration cycles under stringent sanitizing conditions, and exhibits a high binding capacity (Waugh, 2005).

Impressive progresses in recombinant protein technology over the past decades have brought hundreds of therapeutic proteins into clinical applications. As there are hundreds more therapeutic proteins under clinical trials, research aimed to improve this technology will continue at a fast pace (Chen, 2011). However, as the number of high-throughput structural genomics projects increases, the reported percentages of soluble heterologous proteins expressed in *E. coli* has continued to decrease. Although eukaryotic expression hosts are sometimes able to overcome these problems, they are not without their own difficulties in terms of ease of use, time, cost and experimental flexibility (Esposito and Chatterjee, 2006). Nevertheless, *E. coli* expression system continues to dominate the bacterial expression systems and remain the first choice for laboratory investigations and initial development in commercial activities or as a useful benchmark for comparison among various expression platforms (Chen, 2011).

1.8 The discovery of a remarkable polypeptide termed Blad

Germination of leguminous seeds is accompanied by metabolism of the reserve proteins stored in the cotyledons. β-Conglutin, the major globulin component of *Lupinus albus* and a member of the vicilin family of storage proteins, is composed of 10 to 12 major types of subunits (15-72 kDa) and a considerable number of minor subunits, with no disulfide bonds (Ferreira *et al*., 1995a). Approximately half the subunits are glycosylated (Ramos *et al*., 1997). Despite the large heterogeneity observed among different *Lupinus* species in what concerns the polypeptide composition of β-conglutin, this protein suffers an identical fate during germination in all species examined. Between days 3 and 5 following the onset of germination, β-conglutin undergoes a dramatic change in its structure and concentration, involving the appearance of a new set of polypeptides (Monteiro *et al*., 2010).

While studying the changes in ubiquitin and ubiquitin-protein conjugates during seed formation and germination, Ferreira and colleagues (Ferreira *et al*., 1995b) noticed an abrupt accumulation of an abundant 20 kDa polypeptide, hereby named Blad (from the Portuguese/Latin *Banda de Lupinus Albus Doce*, meaning "band from sweet *Lupinus albus"* and referring to a polypeptide band in an electrophoresis gel), in *L. albus* cotyledons during the 4th day after the onset of germination, which is maintained in these organs in high amounts until the 12th day, and then rapidly declines to undetectable levels (Ferreira *et al*., 1995b).
Blad is isolated during growth of *Lupinus* seedlings and is a stable and intermediary breakdown product of β-conglutin catabolism. It is an edible 20 kDa polypeptide, which occurs naturally as part of a soluble 210 kDa oligomer, hereby termed Blad-containing oligomer (BCO), composed of 173 amino acid residues, non-glycosylated but phosphorylated. It is encoded by an internal fragment of the gene encoding the precursor of β-conglutin from *Lupinus* and may be found in all *Lupinus* species examined to date, but in no other legume tested. Blad appears to be extremely basic (pI between 9 and 10) and does not include any cysteine, methionine or tryptophan residues, but contains an extremely high proportion of the nitrogen-rich amino acids, notably arginine (18 residues out of 173), asparagine (17 residues), glutamine (11 residues) and lysine (7 residues) (Monteiro *et al*., 2010). The lack of sulfur containing amino acids comes as no surprise, if we take into account that legume seed storage proteins are notoriously poor in these amino acids (Monteiro *et al*., 2010). Blad also exhibits extreme resistance to denaturation but is extremely sensitive to cleavage, either chemical or proteolytic.

Purification of BCO revealed that it is composed of several polypeptides, the major ones with molecular masses of 14, 17, 20 (i.e. Blad, by far the most abundant), 32, 36, 48 and 50 kDa. The 20 kDa polypeptide is the only with lectin activity, but there is strong evidence that there is a high degree of structural similarity among them all (Ramos *et al*., 1997). Regarding the origin of Blad, the results of an autoradiogram after exposure of 8 days old cotyledons from *L. albus* to [^{35}S] methionine suggested that this polypeptide is not synthesized *de novo*. Several studies also showed that the pattern of accumulation of Blad during germination and seedling growth is an intrinsic characteristic of cotyledons, with no hormonal signal produced by the embryo axes or a light-dependent mechanism involved, but is a characteristic exclusive of the cotyledons from germinating plants of the *Lupinus* genus. In other words, detached cotyledons imbibed in water also produce Blad. It is also interesting to correlate the timing of accumulation of Blad with the corresponding stage of plant development. During the first 4 days after the onset of germination the seedlings are still below the ground and so are naturally protected from most environmental stresses. After 14 d of germination the plantlets possess a number of fully operational leaves and cotyledons photosynthesizing actively to assist rapid plant development – the chances of plant survival are now considerable higher (Ramos *et al*., 1997).

BCO shows a huge potential due to its remarkable multifunctional nature. Its inherent, potent and wide spectrum antifungal activity is responsible for BCO equal or better performance on a large variety of fungal pathogens, when compared to the top chemical fungicides. This has been widely demonstrated by certified companies over the last years (Table 1.1). There are literally thousands of antifungal proteins and peptides described in the literature, with some of them protected by patents. However, as far as we are aware, none has made it to application, either in human health or agriculture.

Table 1.1. In vitro antifungal activity of BCO against different filamentous fungi, yeasts and bacteria.

Plant Fungal pathogens	*In vitro* activity	*Field trials*
Alternaria alternata	+	+
Botryosphaeriaceae	+	
Botrytis cinerea (grapevine, strawberry, tomato)	+	+
Colletotrichum acutatum (soya bean)	+	+
Colletotrichum gloeosporioides (olive)	+	+
Colletotrichum kawahae	+	
Eutypa sp.	+	
Fusarium graminearum	+	
Fusarium oxysporum	+	
Macrophomina phaseolina	+	
Mycosphaerella fijiensis	+	
Pestalotiopsis sp.	+	
Phaeoacremonium sp.	+	
Phaeomoniella chlamydospora	+	
Phomopsis sp.	+	
Verticillium alboatrum	+	
Verticillium dahliae	+	
Eryshiphe necator (grapevine)		+
Leveillula taurica-LEVETA (tomato)		+
Erysiphe cichoracearum (cucumber)		+
Sphaerotheca macularis (strawberry)		+
Podosphaera aphanis (strawberry)		+
Colletotrichum fragariae (strawberry)		+
Monilinia taxa (almonds)		+
Monilinia fructigena (almonds)		+

Animal bacterial pathogens/ Food poisoning microorganisms	*In vitro* activity
Aspergillus niger	+
Pseudomonas aeruginosa	+
Listeria monocytogenes	+
Bacillus subtilis	+
Staphylococcus aureus	+
Salmonella thyphimurium	+

Food spoilage fungi	*In vitro* activity
Aspergillus niger	+
Yarrowia lipolytica	+
Saccharomyces cerevisiae	+
Zygosaccharomyces rouxii	+
Dekkera bruxellensis	+
Kluyveromyces marximianus	+
Zygosaccharomyces bailli	+
Penicillium sp.	+

Animal fungal pathogens	*In vitro* activity
Aspergillus fumigatus	+
Candida albicans	+
Candida dubliniensis	+
Candida glabrata	+
Candida lusitaneae	+
Candida parapsilosis	+
Candida tropicalis	+
Cryprococcus neoformans	+

According to FRAC, when a potential new fungicide is identified, its activity is assessed under laboratory and glasshouse conditions on different fungal pathogens, and is then tested in field trials against an appropriate range of crop diseases in different regions and countries. Only if it works uniformly well against important crop diseases in a large number of trials over several seasons is it considered for development and marketing (Brent and Hollomon, 1995). BCO is already on sale in some countries (USA, Canada and South Korea) for agricultural applications under the tradename Fracture®. Fracture® efficacy equals the best in class synthetic fungicides and, as a bio-fungicide, offers several advantages over its synthetic competitors. Moreover, FRAC classifies each fungicide regarding the risk of development of pathogen resistance to its active ingredient. Resistance risk indicators include, e.g., single site of action in the target fungus, cross-resistance with other fungicides or use of repetitive treatments. This classification is used to establish fungicide rotation programs that can help extend the life and efficacy of each one of them. Because BCO has a completely new, multi-target mode of action, providing decisive, multi-site control in a way that no other fungicide can claim, FRAC included it in a new category, M12, of the 2016 issue of its Code List©. In this way, Blad separates itself from all other fungicides with unknown cross-resistance with any of other fungicide class, making it a key product in disease management programs.

The development of BCO based bio-fungicides fits in the concept of sustainable agriculture based upon the use of production factors from clean technologies that aim at obtaining safe food and raw materials. It is a real alternative to existing chemical fungicides because, unlike most existing biopesticides, does not have serious limitations of applicability, it is a cost-effective solution and, due to its broad spectrum, can replace chemical fungicides to protect crops from virtually all type of pathogens.

1.9 Objectives

The main goal of this research work was to perform the necessary studies to assess the viability of BCO as a candidate to be used in the treatment of human fungal infections and as a plant protection active ingredient. To achieve this objective, three major research lines were conducted:

- Demonstrate the inherent, potent and wide spectrum antifungal activity of BCO and assess its potential to be used as a new antifungal active ingredient by performing *in vitro,* greenhouse and field trials against an array of phytopathogenic fungi, as well as test its *in vitro* efficacy against a variety of human pathogenic yeasts and analyze the kinetics of cell-killing. In addition, an array of toxicological studies to guarantee BCO absence of toxicity towards mammalian cells and pollinators was also conducted.

- Elucidate BCO's complex and multitarget mode of action by (i) investigating the biochemical properties and catalytic activities that allow BCO to target fungal cells, (ii) studying its cellular targets and assessing its effect on the morphology of the cells, (iii) determining the exact mechanism by which BCO causes cell death, and (iv) performing a full structural characterization of Blad aiming the determination of its three-dimensional structure.

- Develop a "super-Blad" with both antifungal and antibacterial activities. Blad has some antibacterial activity specially against food poisoning bacteria, however, the final goal was to develop a novel Blad-based therapeutic with enhanced bacterial activity but also

with the ability to inhibit the growth of phytopathogenic fungi. For that, a new chimeric protein consisting of Blad polypeptide and a selected peptide, SP10-5 or Sub5, known to possess biological potential as an antibacterial agent, was created, thus enabling the simultaneous treatment of fungal and bacterial infections with a single drug in plants.

1.10 References

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Chapter 2

A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts

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Chapter 2 - A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts

Abstract

There are literally hundreds of polypeptides described in the literature which exhibit fungicide activity. Tens of them have had attempted protection by patent applications but none, as far as we are aware, have found application under real agricultural conditions. The reasons behind may be multiple where the sensitivity to the Sun UV radiation can come in first place. Here we describe a multifunctional glyco-oligomer with 210 kDa which is mainly composed by a 20 kDa polypeptide termed Blad that has been previously shown to be a stable intermediary product of β-conglutin catabolism. This oligomer accumulates exclusively in the cotyledons of *Lupinus* species, between days 4 and 12 after the onset of germination. Blad-Containing Oligomer (BCO) reveals a plethora of biochemical properties, like lectin and catalytic activities, which are not unusual *per si*, but are remarkable when found to coexist in the same protein molecule. With this vast range of chemical characteristics, antifungal activity arises almost as a natural consequence. The biological significance and potential technological applications of BCO as a plant fungicide to agriculture, its uniqueness stems from being of polypeptidic nature, and with efficacies which are either equal or greater than the top fungicides currently in the market are addressed.

Key-words: *Lupinus,* polypeptide, antifungal activity, biochemical properties, field trials

2.1 Introduction

Attempted infection of a plant by a fungal pathogen may be compared to a warfare whose major weapons are proteins derived from both plant and pathogen (Ferreira *et al.*, 2006). Plant resistance is determined by an impressive combination of both constitutive and inducible defense mechanisms that involve a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack (Boyd *et al.,* 2013). For example, the completion of the *Arabidopsis* genome sequence showed that this plant species has a few hundred open reading frames that encode potential surveillance proteins (Austin and Ballaré, 2014).

Antifungal proteins, as their name imply, serve a protective function against fungal invasion. They are involved in both constitutive and induced resistance to fungal attack and are produced by a multitude of organisms (Hegedus and Marx, 2013; Ng, 2004). Hundreds of antifungal proteins have been described, with more being discovered almost daily. C. P. Selitrennikoff, (2001) considered thirteen families of antifungal proteins, which were named primarily on the basis of their mechanism of action (e.g. chitinases, β-glucanases), their structure (e.g. glycine rich), or their similarity to a known type of protein (e.g. thaumatin-like protein) (Theis and Stahl, 2004). Several proteins may be classified into more than one family and some are included in the group of pathogenesis-related (PR) proteins.

In plants, the chitin-binding proteins, one family of antifungal proteins, are usually divided in two classes: class I proteins contain a chitin-binding domain similar to a domain present in hevein, a protein from rubber latex (Slavokhotova *et al.,* 2014); class II proteins lack the chitin-binding hevein domain (Theis and Stahl 2004; Slavokhotova *et al.,* 2014). The antifungal activity of chitin-binding proteins is mainly due to their ability to bind fungal cell wall chitin, which results in disruption of cell polarity and consequent inhibition of growth by mechanisms that have not been elucidated (Patil *et al.,* 2013). The antifungal activity displayed by chitinases, another family of antifungal proteins, was originally assumed to derive from their ability to catalytically cleave chitin, leading to a weakened fungal cell wall and subsequent cell lysis. However, more recent evidence indicates that the mechanisms by which chitinases inhibit fungal growth seem to be more dependent on the presence of a chitin-binding domain than or chitinolytic activity (Patil *et al.,* 2013).

Vicilins comprise a major group of legume seed storage globulins. Together with legumins, they usually account for approximately 80% of the total protein in mature legume seeds (Casey *et*

al., 1986). Vicilins are oligomeric proteins (150 to 170 kDa) with variable degrees of glycosylation, composed of three similar subunits of ~40 to 70 kDa, with no disulphide linkages and stabilized by non-covalent forces (Casey *et al.,* 1986; Shutov *et al.,* 1995). The combination of multiple structural genes and extensive posttranslational processing, results in a high degree of subunit polymorphism for these proteins (Higgins, 1984). Nevertheless, vicilins from different legume seeds exhibit a considerable amount of sequence homology and similar 3-D structures (Argos *et al.,* 1985; Lawrence *et al.,* 1990; Ko *et al.,* 1995). Vicilins isolated from a variety of legume seeds have been reported to bind strongly to chitin, chitosan and fully acetylated chitin (Firmino *et al.,* 1996; Sales *et al.,* 1996; Yunes *et al.,* 1998). These interactions were proposed to explain their detrimental effects on the development of the cowpea weevil (*Callosobruchus maculatus*), a bruchid insect that is a pest of cowpea seeds (Yunes *et al.,* 1998; Oliveira *et al.,* 2014) as well as their interference with the germination of spores or conidia of phytopathogenic fungi (Gomes *et al.,* 1997).

Recently, we described a new polypeptide, named Blad, a highly processed multifunctional product of *Lupinus* vicilin gene with a unique biosynthetic route (Monteiro *et al.,* 2010). Blad polypeptide is the major subunit of a glyco-oligomer, here termed Blad-Containing Oligomer (BCO), accumulates exclusively in the cotyledons of *Lupinus* species, between days 4 and 12 after the onset of germination. In this work, a considerable number of differential properties and physiological roles played by this remarkable oligomer at a very specific stage of *Lupinus* development were elucidated. A number of characteristics, deriving directly from the oligomer properties, highlight the potential technological significance of its application. In addition, the uniqueness of BCO stems from being of polypeptidic nature, edible and with efficacies which are either equal or greater than the chemical, usually highly toxic, top fungicides currently in the market.

2.2 Materials and Methods

2.2.1 Ethics statement. All necessary permits were obtained for the described field studies. All the field trials were performed in field trial lands owned by the research companies (Two bees Agric. Research, Synthec Research, FMC Ag. Products Group, Helena R&D, Crop Science and Pan Am. R&D) and specifically maintained for this purpose. In addition, the field studies did not involve endangered or protected species; they were performed with commercial available fruit varieties and with industrial-standards as controls.

The bee colonies were inspected periodically according to good bee keeping practice by an experienced apiarist. Furthermore, the colonies were examined for a reportable bee epidemic by an authorized bee specialist, without any negative findings.

2.2.2 Biological material and growth conditions. Dry seeds of white lupin (*Lupinus albus* L.) cv. Leblanc were obtained from a local market. When appropriate, the seeds were germinated for periods up to 10 days (Monteiro *et al.,* 2010). In all cases, the seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at −80°C until needed.

The majority of the fungal strains tested for Minimum Inhibitory Concentration (MIC) determination were purchased from reference culture collections (American Type Culture Collection—ATCC, and Centraalbureau voor Schimmelcultures— CBS): *Alternaria alternata* (CBS 154.31), *Cercospora zeae-maydis* (ATCC MYA-725), *Colletotrichum acutatum* (CBS 294.67), *Colletotrichum gloeosporioides* (CBS 119204), *Colletotrichum dematium* var. *trucatum* (ATCC 76264), *Colletotrichum graminicola* (CBS 130836), *Exserohilum turcicum* (ATCC 64836), *Fusarium graminearum* (CBS 184.32), *Fusarium oxysporum* (CBS 114750), *Macrophomina phaseolina* (CBS 205.47), *Mycosphaerella fijiensis* (CBS 116635 and 120258), *Sclerotinia sclerotiorum* (CBS 128069), *Verticilium dahliae* (CBS 110277 and CBS 110275) and *Verticilium alboatrum* (CBS 385.91). Two strains isolated in our lab were also used: *Botrytis cinerea* (isolated from tomato), and *Sclerotinia sclerotiorum* (isolated from lettuce). These strains were identified by sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (PCR amplification with primers ITS1 and ITS4). For preparing the inocula, all fungi were grown on Sabouraud Dextrose Agar for 7 days at 25°C.

The honey bee test was carried out with young adult worker bees deriving from a healthy colony. One day before the start of the test, the bees were collected randomly from the outer combs of the colony for the oral and contact toxicity test. The hive used for the honeybee

collection for the test was adequately fed, healthy and as far as possible disease-free and queenright. During the experimental phase the test organisms were kept in constant darkness except at the start of the experimental phase in the oral toxicity test (feeding of the bees) and during the three assessments (4, 24 and 48 h after test start). Feeding, application and assessments were made under neon light.

2.2.3 Purification of proteins. Total globulins from *Lupinus* dry seeds were extracted and isolated as described by (Monteiro *et al.,* 2010). The globulins were subsequently precipitated by the addition of ammonium sulfate (561 g/L), centrifuged at 30,000 *g* for 20 min at 4°C, resuspended in 50 mM Tris-HCl buffer, pH 7.5 (5.7 mL/g of cotyledon) and desalted on PD-10 columns previously equilibrated in the same buffer. For the germinated seedlings, the cotyledons were ground and homogenized with a mortar and pestle in water (pH adjusted to 8.0) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (2 mL/g fresh weight) incubated at 4°C for 30 min with agitation, filtered through cheesecloth and centrifuged at 30,000 *g* for 1 h at 4°C. The precipitate was suspended in the globulin solubilizing buffer (2 mL/g fresh weight; 100 mM Tris-HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)) agitated during 30 min at 4°C and followed by a centrifugation step for 1 h at 30,000 *g* and 4°C and desalted on PD-10 columns. For individual globulins purification, the total fraction was fractionated and purified by AKTA anion exchange chromatography on a Q-Sepharose column (Ø = 1 cm; h = 8 cm; flow rate = 1.5 mL/min) essentially as described by (Monteiro *et al.,* 2010). The bound proteins were eluted with a gradient of NaCl (0 to 1M) and desalted into 50 mM Tris-HCl buffer, pH 7.5. α- and γ-conglutins were purified from *L. albus* cotyledons as reported by (Santos *et al.,* 1997).

BCO was extracted and isolated from the cotyledons of eight-days old seedlings. The protein corresponding to β-conglutin was purified by AKTA anion exchange chromatography as explained above and subsequently subjected to AKTA gel filtration on the Superose 12 HR 10/30 column previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5. This last purification step does not affect the polypeptide pattern of the oligomer, but removes unidentified low molecular mass compounds.

For the field trials studies, the germinated cotyledons were ground and homogenized in a pilot scale hammer mill in water containing 10 mM EDTA and centrifuged at 9,000 *g* and 4°C. The supernatant was heated at 60°C during 1 h and submitted to a new centrifugation step at the

same conditions. Finally, the extract was concentrated in a speed-vacuum evaporator until it reaches 20% (w/v) of BCO.

Lemna ribulose bisphosphate carboxylase was purified as described by (Santos *et al.,* 1997), by a procedure involving extraction of the total soluble protein, AKTA anion exchange chromatography on the Mono Q HR 5/5 column, and AKTA gel filtration on the Superose 6 HR 10/30 column.

2.2.4 Electrophoresis, immunoblotting and production of anti-Blad polyclonal antibodies. One-dimensional, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and immunoblotting were performed as described before (Monteiro *et al.,* 2001). For anti-Blad polyclonal antibodies production, AKTA purified BCO was subjected to preparative SDS-PAGE (10% (w/v) acrylamide). Total polypeptides were visualized with $CuCl_2$ (negative staining; copper staining) (Lee *et al.,* 1987). Blad polypeptide was sliced and the protein eluted as described before (Melo *et al.,* 1994), desalted on a PD-10 column previously equilibrated with water and utilized to immunize two-month-old, male Wistar rats. Each rat was injected subcutaneously with 0.4 mL of a solution containing 0.125 mg Blad and 0.2 ml complete Freund's adjuvant. To obtain a high titer, three identical booster injections were given every four weeks in complete Freund's diluted 1:10 with incomplete adjuvant. At intervals, blood was collected from the heart and the titer followed by immunoblotting. Total blood was taken from the heart 9 days after the third booster injection. Blood samples were allowed to clot and the serum was collected and stored frozen at -80°C. Antibody specificity was thoroughly assessed.

2.2.5 Assays for protein phosphorylation and protein glycosylation. Detection of phosphoryl groups in proteins was performed according to the method described by (Schulenberg *et al.,* 2003). After SDS-PAGE the proteins in gels were fixed overnight in 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was washed in water and incubated with the fluorescent phosphosensor dye Pro-Q diamond (a phosphoprotein gel stain; Molecular Probes) during 2 h, under slow agitation, in the dark, and subsequently destained in 15% (v/v) propylenoglycol containing 50 mM sodium acetate, pH 4.0. The gel was finally analyzed in a fluorimeter Typhoon 8000 (Amersham; excitation λ = 532 nm; emission λ = 583 nm) and photographed. The same gel was then stained for total protein with Coomassie Brilliant Blue R-250.

Detection of carbohydrate residues in proteins was performed by affinoblotting. Proteins separated by SDS-PAGE and blotted onto a PVDF membrane were probed for the presence of glycopolypeptides essentially by the concanavalin A/peroxidase method developed by (Faye and Chrispeels, 1985), as described before (Ferreira *et al.,* 2003).

2.2.6 Enzymatic assays. BCO was assayed for the activities of β-*N*-acetyl-D-glucosaminidase, chitinase, chitosanase and endo-1,3-β-glucanase. Two hundred µg pure protein was used in each enzymatic assay. The controls used were: (i) substrate and buffer; (ii) purified proteins and buffer. All assays were made in triplicate.

β-*N*-Acetyl-D-glucosaminidase (EC 3.2.1.52) activity was measured essentially as reported by (Nichols *et al.,* 1980). The reaction mixture consisted of 2.5 µmol of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 10 µmol citric acid-sodium citrate, and 200 µg of the purified proteins in 1 mL of 10 mM sodium citrate buffer, pH 5.5. The amount of *p*-nitrophenol released was calculated from a standard curve (0.05 to 0.5 µmol *p*-nitrophenol). Enzyme activity is reported as pmol *p*-nitrophenol released per min per µg protein.

Chitinase (EC 3.2.1.14) activity was measured essentially by the method described by (Nichols *et al.,* 1980). The chitinase assay is based on the colorimetric determination of *N*-acetylglucosamine released from chitin. The substrate for the reaction mixtures consisted of 300 mg finely ground chitin dispersed in 80 mL 0.2 M sodium citrate buffer, pH 5.5. The amount of *N*-acetylglucosamine released was determined from a calibration curve (0.68 to 4.5 µmol *N*-acetyl-D-glucosamine). Chitinase activity was expressed as pmol *N*-acetylglucosamine released per min per µg of protein.

Chitosanase (EC 3.2.1.132) activity was assayed as described by (Boucher *et al.,* 1995). Chitosanase activity was determined by measuring the quantity of reducing sugars generated by hydrolysis of soluble chitosan. The substrate for this assay was chitosan dissolved in 50 mM sodium acetate buffer, pH 5.5. The reducing sugars were determined by reading the optical density at 405 nm, and comparison with a standard curve (0.05 µmol to 0.5 µmol D-glucosamine).

Endo-1,3-β-glucanase (EC 3.2.1.6) activity was quantified essentially by the method of (Abeles and Forrence, 1970). Laminarin as the substrate and dinitrosalicylic reagent (150mL 4.5% (w/v) NaOH added to 440mL solution containing 4.4 g 3,5-dinitrosalicylic acid and 127 g K-Na tartrate.$6H_2O$) were used to measure the reducing sugars released. The amount of glucose released was determined by reading the optical density at 500 nm and the results calculated

from a standard curve (0 to 11 μmol D-glucose). Glucanase activity was expressed in pmol glucose released per min per μg of protein.

2.2.7 Fungal inhibition studies. Minimum inhibitory concentrations (MICs) determination. This susceptibility test was made according to the CLSI (former NCCLS) guideline M38-2A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; CLSI), using broth microdilution method, with small modifications The inoculum suspension was prepared by covering the colonies with approximately 5 mL of sterile 0.9% (w/v) saline (NaCl) with 0.01% (v/v) tween 20. The resulting suspension was transferred to a sterile tube, vortexed for 15 s, and the cell density adjusted to 0.4–5.0 x 10^6 CFU/mL by direct counting of spores using a neubauer chamber. The final inoculum suspension was made by a 1:50 dilution with Potato Dextrose broth medium (pH 7.5), prepared with a double concentration, which resulted in a final concentration of approximately 0.4 x 10^4 to 5 x 10^4 cells per mL. The inoculum size was verified by enumeration of CFU obtained by subculturing on SDA plates. Filamentous fungi inocula (100 μL) were added to each well of the microplate, containing 100 μL of the diluted BCO solution (twofold). Final volume in each well was 200 μL. Amphotericin, fluconazole and itraconazole were also tested using the same inocula, but following the dilution scheme described in M38-2A for each case. The microplates were incubated at 25°C without agitation and examined after 72 hours. The MIC endpoints were the lowest drug concentration that showed absence of growth, as recorded visually. **Percentage of inhibition of radial growth in agar media.** This susceptibility test consists in the assessment of the ability of BCO to inhibit the radial growth of fungi in a specific agar medium. The culture medium was selected as the most sensitive for this type of analysis, and it also contained a low concentration of agar, in order to facilitate the diffusion of the oligomer—Potato Dextrose Agar, with 0.6% (w/v) agar and pH 7.5. The oligomer was added to PDA medium (after autoclaving and before pouring in plates) in order to achieve two different final concentrations (4.5 μM and 9.0 μM). The inocula were transferred from the SDA plates using a sterile scalpel blade, by dissecting small agar squares (1–2 mm) containing mycelium and placing them onto the center of each agar plate (one per plate and mycelium facing up). The diameter of the radial growth of the fungus was measured (mm) after 5 days of incubation at 25°C, and the percentage of inhibition of growth was calculated according to the following formula: [(diameter of radial growth in control—diameter of radial growth in medium containing BCO)/ diameter of radial growth in control] x 100.

2.2.8 Field trials

The total 8 days-old cotyledons *Lupinus* extract, containing 0.95 mM of BCO, was evaluated for control of fruit rot caused by *Botrytis* spp on strawberries and powdery mildew caused by *Eryshiphe necator* on grapes. Treatments varied among the field trials conducted between 2008 and 2012 (Tables 2.1 and 2.2), but common objectives were to determine the field efficacy of BCO using full-season treatments. The evaluations were typically conducted close to harvest. In Tables 2.1 and 2.2, treatment descriptions indicate the fungicides used, the location of the trial and the rate of active ingredients with spray-intervals according to phenological timmings. As standards-control, common commercially available fungicides were used and are also described in the tables.

Table 2.1. Strawberry fruit rot (Botrytis cinerea) field trial treatment description.

Trial	Year	Treatment description [a]	Fungicide [b]/rates	Evaluation [c]
1	**2008**	Helena R&D, Watsonville, CA, USA (3 applications@10 day spray intervals)	Non- treated BCO (470g/ha) BCO (650g/ha Elevate (840 g ai/ha)	**11 April** 8 DAA3
2	**2011**	Crop Science, Aromas, CA, USA (5 applications@10-12 day spray intervals)	Non- treated BCO (470g/ha) BCO(650g/ha Pristine (494g ai/ha)	**16 September** 11 DAA5
3	**2011**	Pan Am. R&D, Reno, GA, USA (6 applications@7 day spray intervals)	Non- treated BCO (550g/ha) BCO (740g/ha Endura (450 g ai/ha)	**3 June** 7DAA6
4	**2012**	Hillsboro, OR, USA (5 applications@7 day spray intervals)	Non- treated BCO (450g/ha) BCO (560g/ha Switch (780 g ai/ha)	**18 June** 7DAA5

[a] Treatments were replicated 4 times. Plot area was 1 bed (2 arrows of plants) x 9 m. Plots was arranged in a randomized complete block.

[b] Standards-industrial fungicides use: Elevate – active ingredient: 50% Fenhexamid; Prisitine – active ingredient: 12.8% Pyraclostrobin and 25.2% of Boscalid; Endura – active ingredient: 70% Boscalid; Switch – active ingredient: 37.5% Cyprodinil and 25% Fludioxonil.

[c] Evaluation for control was made at harvest. 8DAA3, means that the evaluation was made 8 days after application 3.

For the strawberry *Botrytis* trials, spraying was made with a backpack spray delivering 1000 L/ha. Plot area was 1 bed (2 arrows of plants) x 9 m. Plots was arranged in a randomized complete block. Incidence was evaluated by harvesting the entire plot and calculating the number of infected fruits per 20 fruits. Severity rating was the average severity of the infected berries sampled in each plot. Treatments were replicated 4 times. For trials number 1, 2 and 3, the disease organism was brought into the trial area and artificially inoculated on strawberries twice during the test period. For the grapevine powdery mildew trials (Table 2.2), spraying was made with an air blast sprayer with spray direct towards the grape foliage and bunches, delivering 1000 L/ha. Plot area was 28 m^2, 5 vines per plot, 4 replicates per treatment. The experimental design was a randomized complete block. Incidence was defined as the percentage of grape clusters with powdery mildew within a sample of 20 clusters. Severity was expressed as the percent of berries infected per cluster.

Table 2.2. Grapevine powdery mildew (*Eryshiphe necator*) field trial treatment description.

Trial	Year	Treatment description [a]	Fungicide [b]/rates	Evaluation [c]
1	**2011**	Two bees Agric. Research, CA, USA Carignane (7 applications@10 day spray intervals)	Non- treated	**13 July** 8DAA7
			BCO (540g/ha)	
			BCO (670g/ha	
			Pristine (335 g ai/ha)	
2	**2012**	Las Condes, Santiago, Argentina Cabernet Sauvignon (4 applications@14 day spray intervals)	Non- treated	**5 March** 14DAA4
			BCO (335g/ha)	
			BCO (450g/ha	
			Folicur (170g ai/ha)	
3	**2012**	Madera, CA, USA Cabernet Sauvignon (2 applications@21 day spray intervals)	Non- treated	**2 August** 10DAA2
			BCO (335g/ha)	
			BCO (450g/ha)	
			Prisitne (335 g ai/ha)	
4	**2012**	Hillsboro, OR, USA Pinot Noir (6 applications@14 day spray intervals	Non- treated	**3 August** 14DAA6
			BCO (335g/ha)	
			BCO (450g/ha	
			Pristine (335 g ai/ha)	

[a] Treatments were replicated 4 times on 5 vines/plot.

[b] Standards-industrial fungicides use: Prisitine – active ingredient: 12.8% Pyraclostrobin and 25.2% of Boscalid; Folicur – active ingredient: 20% TEBUCONAZOL.

[c] Evaluation for control was made at harvest. 8DAA7, means that the evaluation was made 8 days after application 7.

2.2.9 Oral and contact acute toxicity studies to the honeybee *Apis mellifera* L. of BCO. This study was conducted at Eurofins Agrosciense Services, GmbH according to the OECD Guideline No. 213 and No. 214 (1998). The study was carried out with the following treatments in the oral and contact toxicity tests: one dose of the test item (BCO); one control in the oral toxicity test (50% (w/w) sugar solution) and one control in the contact toxicity test (tap water); four doses of the reference item (Perfekthion - a.i. dimethoate). At each dose and treatment, respectively, five replicate groups of 10 bees were tested. For the oral toxicity test, BCO was dissolved in tap water to get a stock solution. The test bees were starved for 2 h before they were fed with the solutions. A quantity of 250 µL of test item or reference item solution was offered to each cage of 10 bees to ensure sufficient consumption of test or reference item. The amount of test solution consumed by each replicate was determined by weighing the feeders (Eppendorf cups) before and after feeding. After a period of 6 h the feeding solution was totally consumed by the bees and the feeders were exchanged. During the observation period the bees were supplied ad libitum with untreated 50% (w/v) aqueous sucrose solution. In the control group the bees were fed with 50% (w/v) aqueous sucrose solution for up to 6 h after the 2 h starvation period. For the contact toxicity test, BCO was directly dissolved in tap water so that 2 µL contained the required nominal amount of active ingredient per bee. The reference item was dissolved in tap water in order to get a stock solution at the correct concentration for the highest dose so that 2 µL contained the required nominal amount of reference item per bee. After the bees had been anaesthetized with carbon dioxide they were treated individually by topical application with a micro applicator. Two µL of the control, test and reference item solutions were applied dorsally to the thorax of each bee. Between every application, the outside of the micro applicator needle was cleaned with a mixture of water and a water-wetting agent. After application the bees were returned to the test cages and fed with a 50% (w/v) aqueous sucrose solution ad libitum. In the oral and contact toxicity tests the number of dead bees in the individual test cages was recorded 4, 24 and 48 h after the start of feeding and after application, respectively.

2.2.10 Statistical analysis. In the field trials, the effects of treatments on incidence and severity of both *Botrytis* on strawberry and powdery mildew on grapes were analyzed separately for each trial. A one-way analysis of variance was performed on each dataset, and mean separation ($P<0.05$) was done by Duncan´s New Multiple Range Test.

In the bee studies, the LD_{50} values with 95% confidence limits of the reference item treatment were calculated by means of a probit analysis using the statistical program SASProprietary Software 9.2, (2002–2008). The oral LD_{50} values for the reference item treatment were calculated with the consumption values per replicate.

2.2.11 General assays. Protein content was measured using a modification of the Lowry method (Bensadoun and Weinstein, 1976).

The BCO content of the 8 days-old cotyledons *Lupinus* extract, was quantified by an HPLC method using as standard a calibration curve made with purified BCO.

Affinity-purified, polyclonal anti-ubiquitin antibodies to SDS-treated bovine ubiquitin were produced in rabbits and prepared as described by (Ferreira *et al.,* 1995).

2.3 Results and Discussion

2.3.1 Biochemical characteristics of BCO. The native molecular mass of the native BCO has been estimated by gel filtration as 210 kDa. A simple SDS-PAGE analysis reveals that this oligomeric protein is composed of several polypeptides, the major ones exhibiting molecular masses of 14, 17, 20 (Blad; by far, the most abundant), 32, 36, 48 and 50 kDa (Fig. 2.1A, lanes 1 and 5). Previous studies demonstrated that the oligomer is glycosylated, whereas Blad polypeptide is non-glycosylated. Furthermore, among the oligomer, Blad exhibits lectin activity, as evidenced by its ability to bind to antibodies (i.e. immunoglobulins G) and other glycoproteins (Ramos *et al.,* 1997).

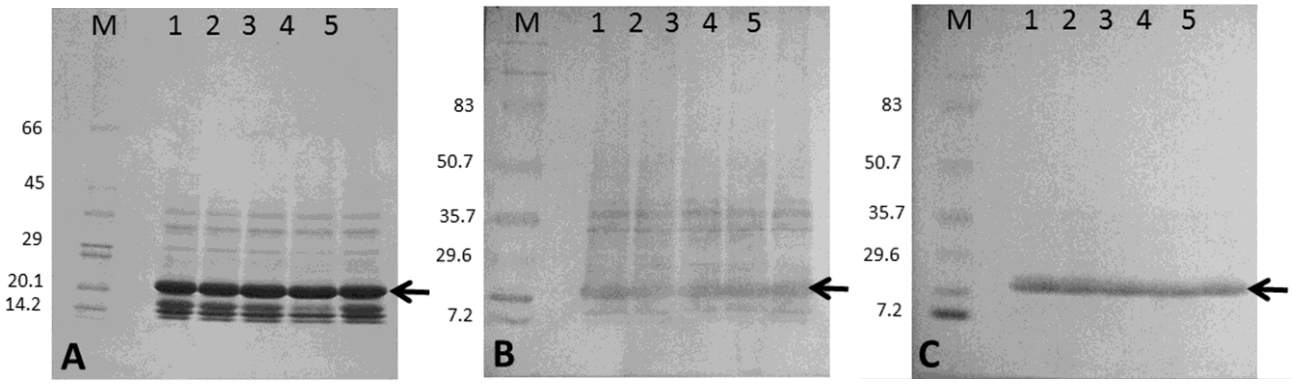

Figure 2.1. Resistance to inactivation of the lectin activity of BCO. BCO was purified, incubated for 10 min at 0°C in the presence of water (control, lanes **1** and **5**), 4 N HCl (lane **2**), 8 N HCl (lane **3**) and 12 N HCl (lane **4**). The protein samples were subsequently analysed by SDS-PAGE and stained for total protein (50 µg per lane; (**A**)) or blotted onto a membrane and probed with anti-blad oligomer antibodies (15 µg per lane; (**B**)) or with affinity purified, anti-ubiquitin antibodies (50 µg per lane; (**C**)). Molecular masses of standards are indicated in kDa.

When BCO, together with the three major storage globulins present in *Lupinus* seeds (i.e. α-, β-, γ-conglutins) were assayed for the presence of phosphoryl groups, the results presented in Figure 2.2 were obtained. Some of BCO sub-units are phosphorylated, as are α-, β- and γ-conglutins sub-units.

The total polypeptides that compose the BCO were resolved by SDS-PAGE (Fig. 2.1A, lane 1), transferred onto a blotting membrane and probed with anti-BCO antibodies. The results obtained, presented in Figure 2.1B, lane 1, reveal structural homologies between Blad and some, but not all polypeptides that constitute the oligomer. Probing an identical membrane

with affinity-purified anti-ubiquitin antibodies indicates that Blad is the only polypeptide exhibiting lectin activity (Fig. 2.1C, lane 1).

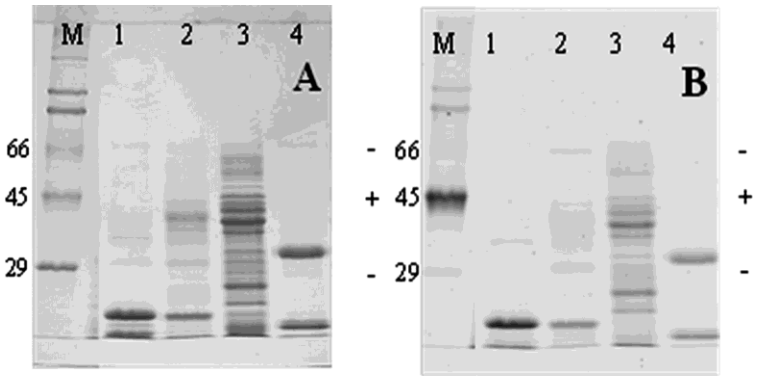

Figure 2.2. Detection of phosphoryl groups in BCO. BCO, α, β, and γ-conglutins were purified from *L. albus*, subjected to SDS-PAGE, and stained for total protein (**A**) or analyzed for the presence of phosphoryl group using the Pro-Q diamond phosphoprotein gel stain (**B**). Lanes 1, 2, 3, 4: BCO, α, β, and γ-conglutins, respectively. Lanes M: molecular mass standards (kDa). The position of the positive (+) and negative (-) phosphorylated markers is shown in B.

The results presented in Figure 2.1 further indicate that the lectin-like activity of Blad is extremely resistant to inactivation, with the oligomer exhibiting a very high stability against denaturation, withstanding prolonged boiling, treatment with organic solvents and detergents, and exposure to high concentrations of strong acids (e.g. 12 N HCl) as is shown in Figure 2.1, lanes 2, 3 and 4. In fact, the only treatments which were found capable of abolishing the lectin activity of Blad were those that induce cleavage of peptide bonds (Ramos *et al.,* 1997). This observation was confirmed by a study on the susceptibility of BCO to proteolysis. To this end, the oligomer was mixed with common proteolytic enzymes, namely pronase, trypsin, proteinase K, α-chymotrypsin and subtilisin, and incubated at room temperature for 1 h, 2 h or 3 h followed by addition of a marker protein (55 μg pure ribulose bisphosphate carboxylase) and a further 1 h incubation. Pure ribulose bisphosphte carboxylase was readily degraded by all proteases in all cases. The results obtained after SDS-PAGE analysis of the incubated reaction mixtures (Fig. 2.3) indicate that the BCO protein is readily hydrolyzed by all proteases tested. This experiment also showed the absence of proteolytic fragments, which could act as proteinase inhibitors or anti-nutritional factors. This conclusion is based on the observation that all proteases tested readily degraded the marker protein added to the reaction medium after the enzymatic digestion of the BCO.

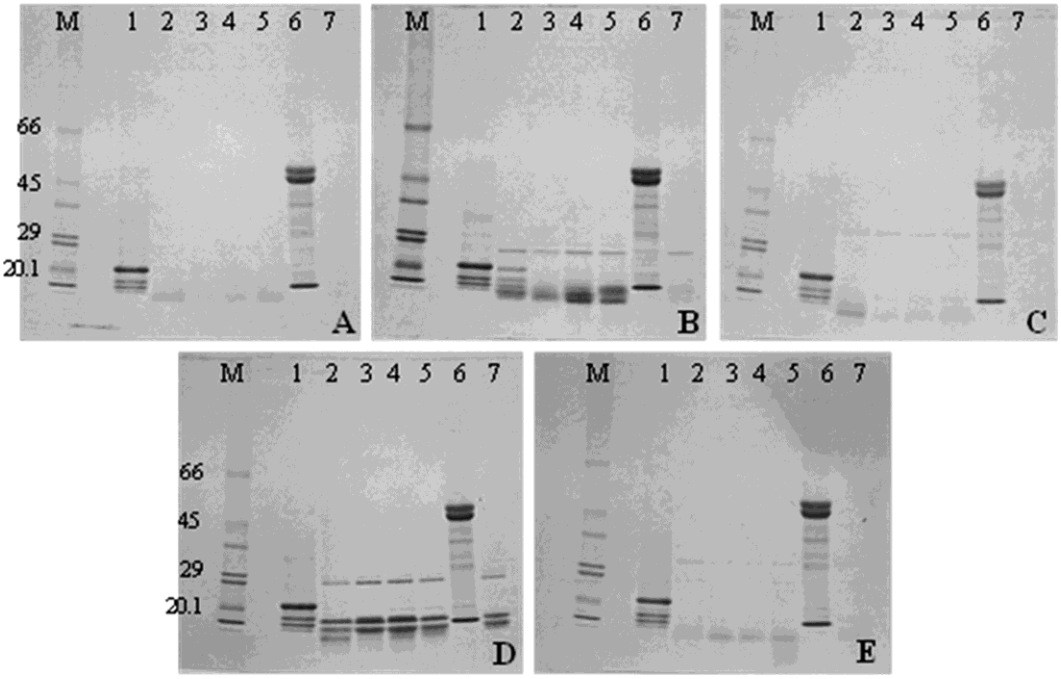

Figure 2.3. Susceptibility to proteolysis of the BCO. Pure 210 kDa protein (lanes **1**) was mixed with proteolytic enzymes [pronase in (**A**); trypsin in (**B**); proteinase K in (**C**); α-chymotrypsin in (**D**); subtilisin in (**E**)] and incubated at room temperature for 1 h (lanes **2**) or 2 h (lanes **3**) followed by addition of pure ribulose bisphosphate carboxylase (55 μg) and a further 1 h incubation (lanes **4**). In lane **5**, pure ribulose bisphosphate carboxylase (55 μg) was incubated for 1 h with the corresponding proteolytic enzyme. Lanes **6** and **7** contain pure ribulose bisphosphate carboxylase (55 μg) or the corresponding protease (20 μg), respectively. Lanes **M**: molecular mass standards (kDa).

2.3.2 Catalytic activities and chitin affinity of BCO. In a subsequent set of experiments, the oligomeric protein was assayed for several enzymatic activities, including those of β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.52), chitinase (EC 3.2.1.14), chitosanase (EC 3.2.1.132), and endo-1,3-β-glucanase (EC 3.2.1.6). BCO was extracted and purified from eight days germinated cotyledons and assayed for catalytic activity as described in the Methods section. Two hundred μg of purified protein was used in each assay. All measurements were made in triplicate. The results shown in Table 2.3 indicate that the BCO exhibits high levels of β-*N*-acetyl-D-glucosaminidase (7.38 ± 0.00487 pmol of *p*-nitropheny released per min and μg of protein) and chitosanase (31.9 ± 1.16 pmol of D-glucosamine released per min and μg of protein) activities but no chitinase or β -1,3-glucanase activities.

Table 2.3. Catalytic activities of BCO.

Enzymatic activity	BCO
β-*N*-Acetyl-D-glucosaminidase	7.38 ± 0.0048[a]
Chitinase	0
Chitosanase	31.9 ± 1.16[b]
Endo-1,3-β-glucanase	0

[a,b] Catalytic activity is expressed as pmol of *p*-nitrophenyl or D-glucosamine, respectively, released per min μg of protein.

In conclusion, these studies showed that in addition to its role as a seed storage protein, BCO binds to immunoglobulins G and other glycoproteins, such as alkaline phosphatase and peroxidase, because it exhibits lectin activity also displaying a very interesting range of enzymatic activities, namely β-*N*-acetyl-D-glucosaminidase and chitosanase activities.

Legume lectin interaction with carbohydrates has long been known to require tightly bound Ca^{2+} and Mg^{2+} (or another transition metal) (Sharon and Lis, 1990). A lectin isolated from the bark of *Sophora japonica* has been reported to be self-aggregatable in the presence of Ca^{2+} and Mg^{2+} due to the binding activities of its four subunits, which enable them to recognize and bind N-linked oligosaccharide chains on three of the four subunits (Ueno *et al.,* 1991).

As for the Lupinus seed storage proteins, including α-, β- and γ-conglutins, BCO undergoes a self-aggregation process in a Ca^{2+}/Mg^{2+}-dependent manner (Ferreira *et al.,* 1999). The simplest explanation would be a self-aggregation of the multivalent lectin activity of the oligomer. However, Ferreira and colleagues (Ferreira *et al.,* 2003) provided evidence that this self-aggregation is electrostatic in nature, rather than lectin-mediated. Bridging of calcium ions between negatively charged protein molecules apparently ensures the supramolecular protein association, in the same way as cross-linking between milk submicelles, where calcium ions form bridges between the negatively charged phosphate groups of α- and β-casein molecules present in adjacent submicelles (Coultate, 1989). In this way, the phosphate groups that are present in BCO, as well as in α-, β- and γ-conglutins (Fig. 2.2), may participate in this process. Presumably, this self-aggregation mechanism could explain the *in vivo* macromolecular aggregation of legume seed storage proteins that ensures their efficient packing inside the protein storage vacuoles.

The observations that BCO displays a lectin-like activity and catalytic activities of β-*N*-acetyl-D-glucosaminidase and chitosanase prompted us to test whether it also binds to chitin. The results presented in Figure 2.4 clearly show that the oligomer does bind in a very strong manner to a chitin column, being eluted with 0.05 N HCl. Indeed, this observation led to the development of a single-step purification method to isolate, in a highly purified manner, the native oligomer. The experiment illustrated in Figure 2.4B shows the single-step, chitin affinity chromatography purification of the BCO. The total globulin fraction isolated from the cotyledons of 8-day old *L. albus* seeds was directly applied to a chitin column. The column was washed thoroughly and the bound proteins eluted with 0.05 N HCl. The gel shown in the Figure 2.4 allows a comparison of the polypeptide pattern eluted from the chitin column with that of the BCO highly purified by the conventional purification procedure.

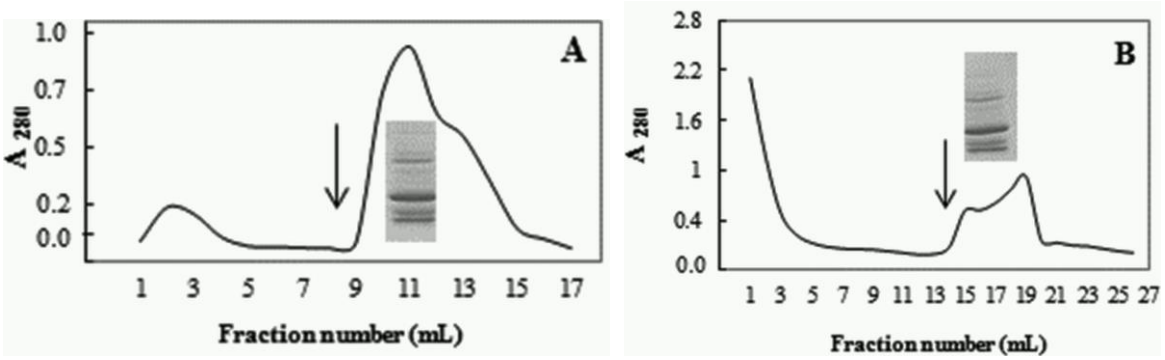

Figure 2.4. Purification of BCO by chitin-affinity chromatography. (**A,B**) Pure BCO (1.2 absorbance units; (**A**)) or the total globulin fraction from 8-days germinated *Lupinus* cotyledons (10 absorbance units; **B**)), respectively, were loaded into a chitin column previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The column was washed and the bound proteins eluted with 0.05 N HCl (beginning of elution is marked with a vertical arrow). One mL fractions were collected. SDS-PAGE analysis of the polypeptide patterns of BCO by the standard, extensive procedure or purified by the single step, chitin-affinity chromatography procedure are illustrated in the figure.

Chitin, an obvious natural ligand of BCO, has never been detected in higher plants (Juliana *et al.,* 2012). However, chitin is an important constituent of the exoskeleton arthropods and nematodes and is present in the midgut of some insects as a component of the peritrophic membrane (Merzendorfer and Zimoch, 2003). Chitin is also the major cell wall component in filamentous fungi and a minor constituent in Oomycetes (Blackwell, 1988). On the other hand, chitosan is another structural polysaccharide found in fungal cell walls (Blackwell, 1988). It is therefore tempting to speculate about the exact physiological role played in *Lupinus* species by the capacity to bind chitin exhibited by BCO.

With all this new-coming information available, and regarding that probably the polypeptide Blad is the main responsible for all this oligomer biochemical characteristic, it becomes increasingly important to work with Blad isolated by conventional biochemical techniques from the other polypeptides that compose the native protein. However, this isolation procedure turned out to be a non-easy task. Besides SDS-PAGE, the only technique that allowed a reasonable isolation of Blad was reverse phase (RP)-HPLC on a C-18 column and even that with a poor level of purification.

2.3.3 In vitro antifungal activity of BCO against phytopathogenic fungi. As stated above, besides functioning as a seed storage globulin, BCO may well fulfil other physiological roles in the plant. This suggestion is based on the considerable number of distinct biochemical properties exhibited by BCO, most notably its catalytic activities, lectin activity and resistance to denaturation. One question naturally emerged at this point of the research—does the oligomer exhibit any anti-biological activity towards those organisms that contain chitin in their structure, namely fungi and insects? To answer this question several experiments were conducted. The susceptibility of several phytopathogenic fungi to the oligomer was assessed in vitro by the determination of the Minimum Inhibitory Concentration, i.e., the lowest concentration of BCO that inhibits the visible growth of a fungal strain. The same strains were tested against three well-known antifungal compounds (amphotericin B, itraconazole, and fluconazole) and the results are shown in Table 2.4.

BCO was the only compound that inhibited the growth of all strains tested. The MIC range was relatively narrow, but it is possible to distinguish some species that seem to be more susceptible, namely, *B. cinerea, Verticillium* spp. and *C. graminicola*. Amphotericin, itraconazole and fluconazole failed to inhibit the complete set of species, although they were tested within the range of concentrations specified in the international standards for this test, and MICs were also within the common values for this type of test (Bruno *et al.,* 2010; Silva *et al.,* 2007). Although the doses of BCO required for fungal inhibition in vitro are higher than those usually required for other antifungal drugs, its molecular weight is also substantially higher (210 kDa), which means that the number of molecules required to exhibit the same inhibitory effect is quite similar between all these drugs. Considering the range of MIC values, and expressing the results in number of molecules, the same inhibitory effect is obtained with: 2.04×10^{13}–$2.60 \times$

10^{15} molecules/mL of amphotericin (MIC 0.034–4.33 μM), 2.67 x 10^{13}–8.53 x 10^{14} molecules/mL of itraconazole (MIC 0.044–1.42 μM), 1.97 x 10^{15}–1.26 x 10^{16} molecules/mL of fluconazole (MIC 3.27–209 μM), and 8.96 x 10^{13}–7.17 x 10^{14} molecules/mL of BCO (MIC 0.15–1.19 μM). Thus, the oligomer seems to have a higher inhibition power by molecule than the azoles and similar to amphotericin. Even if we assume that the polypeptide Blad is the solely molecule responsible for the antifungal activity (molecular weight of 20 kDa), the number of molecules required for inhibition is in the range of 9.40 x 10^{14}–7.53 x 10^{15} molecules/mL, and is, therefore, quite similar to the values observed for azoles.

Table 2.4. *In vitro* susceptibility of phytopathogenic fungi to BCO and other reference antifungal drugs as determined by MIC (Minimum Inhibitory Concentration).

Fungi	MIC (μM)*			
	Amphotericin	**Itraconazole**	**Flucanazole**	**BCO**
Alternaria alternata	1.08 – 2.17	0.71	209	0.30-0.60
Botrytis cinerea	0.54	0.044	6.53	0.15
Cercospora zeae-maydis	0.034	0.35 – 0.71	3.27 – 6.53	0.30
Colletotrichum acutatum	2.17 – 4.33	0.71	>209	0.60
Colletotrichum dematium f. trucatum	4.33	>22.67	>209	1.19
Colletotricum gloeosporioides	4.33	1.42	>209	0.60 -1.19
Colletotrichum graminícola	0.27	0.044	6.53	0.15 – 0.30
Fusarium graminearum	2.17 – 4.33	>22.67	>209	0.30 – 0.60
Fusarium oxysporum	>17.33	>22.67	>209	0.60
Verticillium dahliae	0.135	0.71 – 1.42	13.06	0.15-0.30
Verticillium alboatrum	0.068	0.71	13.06	0.15 – 0.30

* MIC values were obtained from triplicate analysis and different results between replicates are separated by hyphen

Another method used to assess the fungal susceptibility to BCO was the agar dilution method. This method has the advantage of not requiring a standard spore suspension for inoculum, therefore allowing testing strains which have some difficulties in producing spores in culture medium. Furthermore, it mimics the application of the product in plants, where the contact

between the antifungal compound and the fungus takes place on the surface of the leaves. However, given the matrix of the agar and the size of the oligomer, diffusion is also more difficult, resulting in a higher dose required for achieving the same inhibitory effect.

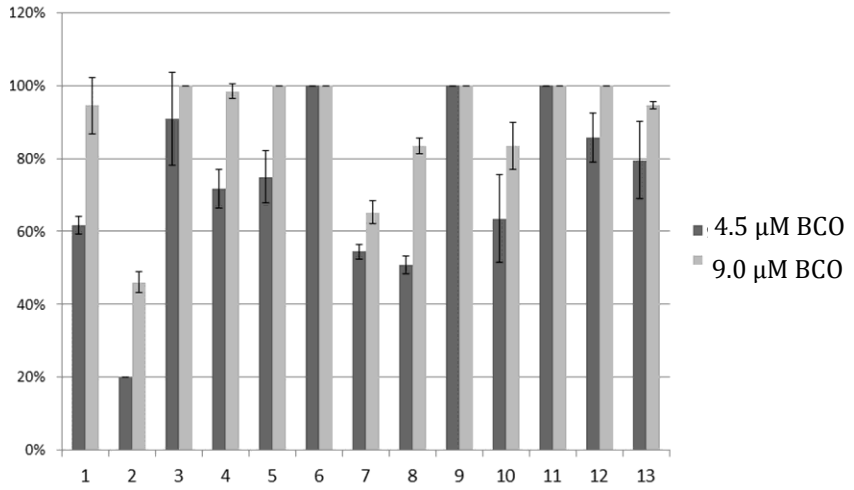

Figure 2.5. Percentage of inhibition of radial growth of several phytopathogenic fungi in an agar medium containing two different concentrations of BCO (4.5 and 9.0 µM). 1: *B. cinerea*, 2: *C. acutatum*, 3: *C. dematium*, 4: *C. gloeosporiodes*, 5: *C. graminicola*, 6: *E. turcicum*, 7: *F. oxysporum,* 8: *F. solani*, 9*: M. fijiensis*, 10: *M. phaseolina*, 11: *S. sclerotiorum* (CBS 128069), 12: *S. sclerotiorum* (CEV—micelium), 13: *S. sclerotiorum* (CEV—sclerotia). Values and error bars represent the mean and standard deviation of triplicate measurements.

The results (Fig. 2.5) clearly indicate that growth inhibition was influenced by the concentration of the oligomer, and that there were species particularly susceptible, namely *C. dematium, E. turcicum, M. fijiensis* and the CBS strain of *S. sclerotiorum*. The next most susceptible group, was more dose dependent, and included *B. cinerea, C. gloeosporioides, C. graminicola*, and the CEV *S. sclerotinia*. The less susceptible strains were *C. acutatum*, followed by *F. solani* and *F. oxysporum*.

As expected from its biochemical properties BCO showed a strong antifungal activity. These effects probably result from the strong chitin-binding capacity of the oligomer (Fig. 2.4). Indeed, the antifungal activity of two important families of antifungal proteins, chitin binding proteins and chitinases, is mainly due to their ability to bind fungal cell wall chitin (Hegedus and Marx, 2013). BCO may therefore be considered as an antifungal compound. Among these, because it is devoid of chitinolytic activity and lacks the chitin-binding hevein domain, it may be classified as a class II chitin binding protein (Theis and Stahl, 2004; Slavokhotova *et al.,* 2014). In addition,

plant chitin binding proteins have been classified as family 4 pathogenesis-related (PR-4) proteins (Slavokhotova *et al.,* 2014).

Several vicilin-like storage proteins from seeds, including cowpea (*Vigna unguiculata*) and other legume species, were shown to bind to chitin (a β-1,4-linked polysaccharide biohomopolymer of *N*-acetyl-D-glucosamine) and to chitosan (deacetylated chitin) (Sales *et al.,* 1996). It has been generally assumed that all chitin-binding proteins for which the amino acid sequence is known contain a common structural motif of 30 to 43 amino acid residues with several cysteines and glycines at conserved positions—this motif is often referred to as the chitin-binding domain (Raikhel *et al.,* 1993). Thus, for example, the vacuolar chitinases of class I possess an N-terminal cysteine-rich domain homologous to hevein and to chitin-binding lectins such as wheat germ agglutinin and *Urtica dioica* lectin (Iseli *et al.,* 1993). However, extensive sequencing of polypeptide fragments of BCO revealed no homologies with chitin-binding domains or the well characterized family of legume lectins, but exhibited an extensive homology with some vicilin-like legume storage proteins, and with α-subunit of β-conglycinin from *Glycine max* in particular (Ferreira *et al.,* 2003). Interestingly, one of Blad polypeptide internal fragments exhibits high homology with a putative disease resistance protein. The data obtained suggest that the lectin activity associated to BCO is unrelated with the so far well characterized legume lectins.

The attack of fungal cell walls by plant chitinases is an important plant defense to fungal infection because it liberates elicitor-active chitin oligomers and weakens the fungal cell wall (van den Burg *et al.,* 2003). However, during invasive growth of hemibiotrophic *Colletotrichum* spp. and biotrophic rust fungi, for example, chitin is exclusively present in the cell walls of exterior infection structures. i.e. germ tubes and appressoria. As a strategy employed by fungi to avoid plant detection and defense, instead of this elicitor-active molecule, the surface of hyphae that grow within host leaves contain chitosan, a deacetylation product of chitin possibly generated by the enzymatic activity of a differentiation induced fungal-chitin deacetylase (El Gueddari *et al.,* 2002). The observation that chitosan lacks elicitor activity led Schulze-Lefert and Panstruga (Schulze-Lefert and Panstruga, 2003) to speculate that the "wolf intrudes in sheep's clothing". Under these circumstances, the penetration hypha could be protected by enzymatic deacetylation unless the plant tissue possesses an efficient chitosanase activity—

BCO may well play an important role here, in the protection of *Lupinus* tissues from fungal invasive growth.

2.3.4 Antifungal activity of BCO against grape *Eryshiphe necator* and strawberry *Botrytis cinerea* under field trials conditions. The reasons behind the fact that none of the antifungal polypeptides described in literature are being used in the agriculture may be multiple where the sensitivity to the Sun UV radiation can come in first place. At this point the research clearly has been demonstrated that BCO has an important activity against plant pathogens in *in vitro* tests. The field conditions are the ultimately step and with utmost importance that could represent the key of success in launching a new natural fungicide to the pesticide market. To address this issue, full-season spray treatments have been performed. Tables 2.5 and 2.6 show the results obtained with the oligomer, in the control of fruit rot caused by *Botrytis* spp. (Table 2.5) on strawberries and Powdery mildew in grapes caused by *E. necator* (Table 2.6), when compared with industry-standards fungicides. Due to the fact that *E. necator* is an obligate pathogen, in vitro studies could not be performed but field trials were included in this study given its economic and environmental burden.

Four trials were conducted in strawberry fruit rot caused by *B. cinerea* (Table 2.5). In trials number 1, 2 and 3, the disease organism was brought into the trial area and artificially inoculated on strawberries twice during the test period. The disease established well and developed moderate to high infection in the plots. The number of applications differed among the trials and also the interval between sprayings. The rate of disease control was compared with different industrial-standards at a commercial rate (Table 2.1. Materials and Method section). All plots were rated for *Botrytis* incidence by picking all ripe berries prior to each application. Percent incidence and severity of disease were evaluated. Against a moderate to heavy infestation of *Botrytis* all the treatments provided statistically significant control of *Botrytis* compared to the untreated control. However, the 8-days old *Lupinus* extract at >650 g Blad/ha was the treatment that provided the best control of *Botrytis*, equal or better than the industrial-standards. There was no phytotoxicity to foliage, flowers or fruits from the *Lupinus* extract.

Table 2.5. Strawberry Botrytis fruit rot (*Botrytis cinerea*) incidence and severity in field trials experiments.

Trial	Treatment*	Incidence (%)§ on clusters	Severity (%)§ on clusters
			8DAA7 ϕ
	Non-treated control	42.0a¥	2.0c
1	BCO (470 g/ha)	27.8a	4.8b
	BCO (650 g/ha)	17.5b	3.0c
	Elevate (840g ai/ha)	10.5b	6.8b
			11DAA5
	Non-treated control	46.3a	29.2a
2	BCO (470 g/ha)	18.8b	11.5b
	BCO (650 g/ha)	15.8b	7.1b
	Pristine (494g ai/ha)	16.3b	22.6a
			7DAA6
	Non-treated control	45.0a	38.3a
3	BCO (550 g/ha)	0.5b	0.13b
	BCO (740 g/ha)	0.0b	0.0b
	Endura (450g ai/ha)	0.0b	0.0b
			7DAA5
	Non-treated control	22.0a	17.2a
4	BCO (450 g/ha)	7.0b	7.0b
	BCO (560 g/ha)	4.0b	2.0c
	Switch (780g ai/ha)	5.0b	0.5c

* Treatment conditions are described in Table 2.1.
§ Incidence was evaluated by harvesting the entire plot and calculating the number of infected fruits per 20
fruit. Severity rating was the average severity of the infected berries sampled in each plot.
ϕ DAA—means days after application.
¥ Means followed by the same letter do not significantly differ (P = 0.05, Duncan´s New MRT). Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Four Powdery mildew grape trials are reported (Table 2.6). The number of foliar applications differs among trials but all sprayings were made from pre-bloom trough fruit development. BCO was applied at rates between 335–670 g/ha and compared with Pristine at 335 g/ha (trials 1, 3 and 4) or with Folicur at 170 g/ha (trial 2). In trials number 1 and 3, disease was late developing but pressure became intense as the season progressed, with roughly 100% incidence on fruit during July. In all trials both rates of BCO as well as the industrial-standards held the powdery mildew infestation and severity of infection to low levels through the rating period. The treated plots for all treatments had significantly less powdery mildew than the untreated control. Treatments with BCO were as effective as or better than (P.0.05) the

industrial-standards in reducing incidence and severity in the experiments conducted in 2011 and 2012.

Table 2.6. Grapevine Powdery Mildew (*Erysiphe necator*) incidence and severity in field trials experiments.

Trial	Treatment*	Incidence (%)§ on clusters	Severity (%)§ on clusters
			8DAA7 ϕ
	Non-treated control	92.8a¥	21.8b
1	BCO (540 g/ha)	58.6a	7.8b
	BCO (670 g/ha)	24.8b	18.5b
	Pristine (335 g ai/ha)	10.5b	8.8b
			14DAA4
	Non-treated control	50.9aa	39.3a
2	BCO (335 g/ha)	2.7c	3.3c
	BCO (450 g/ha)	5.1c	3.1c
	Folicur (170 g ai/ha)	27.3b	22.6b
			10DAA2
	Non-treated control	100.0a	Non rated
3	BCO (335 g/ha)	40.4b	Non rated
	BCO (450 g/ha)	30.6b	Non rated
	Pristine (335 g ai/ha)	55.2b	Non rated
			14DAA6
	Non-treated control	60.0a	34.5a
4	BCO (335 g/ha)	3.0b	4.8b
	BCO (450 g/ha)	2.3b	1.0b
	Pristine (335 g ai/ha)	2.0b	0.5b

* Treatment conditions are described in table 2.2.
§ Incidence was defined as the percentage of grape clusters with powdery mildew within a sample of 20 clusters. Severity was expressed as the percent of berries infected per cluster.
ϕ DAA – means days after application.
¥ Means followed by the same letter do not significantly differ (P=0.05, Duncan´s New MRT). Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

2.3.5 Oral and contact toxicity of BCO to the honeybee *Apis mellifera* L. In a final experiment, due to the nowadays toxicity concerns with fungicides in honeybees population and given the catalytic activities of the oligomer, the toxicity of the BCO was tested. The oral and contact toxicity tests were assessed in young adult worker bees derived from a healthy colony which descended from a breeding line of a beekeeper in Rheinland-Pfalz, Germany. Bees were exposed to the oligomer by feeding and by topical application. The test was done during 48 h with 5 replicates, each composed of 10 bees in one cage per test concentration. Mortality

was assessed after 4, 24 and 48 h. In case of symptoms of poisoning, the behavioural differences between the bees of the control group and those of the test item treatment were noted at each observation interval. In the oral toxicity test, the bees of the control group received an aqueous sucrose solution with a final concentration of 500 g/L (50% (w/v)). In the contact toxicity test, the bees of the control group were treated with tap water. As reference item "Perfekthion" (active ingredient- dimethoate) was used in the oral and contact toxicity test. Four geometrically spaced doses of the reference item were tested.

Table 2.7. *Apis mellifera* L. mortality and total consumption in the oral toxicity test in the control, the test item BCO and reference item groups.

Treatment (Target dose)	**Test item consumed**	**Mortality [%]**	
		24 h	**48 h**
Control (sugar solution)	--	0.0	0.0
	Test item: BCO (µg/bee)		
100	109.42	0.0	0.0
	Reference item: Perfekthion (µg/bee)		
0.06	0.07	0.0	2.0
0.08	0.09	8.0	18.0
0.11	0.12	62.0	68.0
0.15	0.18	92.0	94.0

Table 2.7 shows the results of the oral toxicity test, which was carried out with the target dose level of 100 µg BCO/bee. The actual consumption per bee in the oral test was 109.42 µg BCO/bee. The reference item was tested with the nominal dose levels of 0.06, 0.08, 0.11 and 0.15 µg a.i./bee. The mortality in the oral toxicity test is given as a function of the target dose and actual dose of BCO or reference item and test solution consumed, respectively. Table 2.8 shows the results of the contact toxicity test, which was carried out with the target dose level of 100 µg BCO/bee. The reference item was tested with the nominal dose levels of 0.10, 0.15, 0.23, 0.34 µg a.i./bee. The LD_{50} values of BCO in both tests are presented in Table 2.9. No mortality occurred in the control groups of the oral and contact toxicity tests during the 48-hour observation period. The 24-hour oral and contact LD_{50} values for the reference item were 0.12 and 0.23 µg dimethoate/bee, respectively. Consequently, validity criteria for both control and reference item mortality were met and the test was deemed valid.

Table 2.8. *Apis mellifera* L. mortality in the contact toxicity test in the control, the test item BCO and reference item groups.

Treatment	Mortality (%)	
	24 h	**48 h**
Control (Tap water)	0.0	0.0
Test item: BCO (µg/bee)		
100	0.0	0.0
Reference item: Perfekthion (µg ai/bee)		
0.10	0.0	0.0
0.15	16.0	26.0
0.23	68.0	72.0
0.34	66.0	66.0

Table 2.9. LD_{50} values in the *Apis mellifera* L. oral and contact toxicity tests with BCO.

BCO [µg/bee]			
24 h		**48 h**	
LD_{50}	**Limits** [a]	**LD_{50}**	**Limits**
Oral toxicity test			
> 109.42	-	> 109.42	-
Contact toxicity test			
> 100	-	> 100	-

[a] Lower and upper confidence limits, (95% Confidence interval);

$p \leq 0.05$

From a biological point of view, BCO may play one or more physiological roles in increasing the chances of plant survival in addition to its function as a storage protein. Indeed, the oligomer appears to reunite in a single molecule selected characteristics of legume seed storage proteins, lectins, antifungal proteins and PR proteins, making it a versatile multifunctional protein—it is a seed storage protein with lectin activity, exhibiting catalytic activities of chitosanase and β-*N*-acetyl-D-glucosaminidase, and the capacity to bind in a strong manner to chitin. It is extremely resistant to chemical inactivation but readily degraded by proteolytic enzymes. These biochemical characteristics explain its intense antifungal properties. Furthermore, the biological properties of the oligomer account for its strange pattern of formation and accumulation in the cotyledons during the germination of *Lupinus* seeds. The oligomer, an intermediate breakdown product of β-conglutin catabolism, abruptly accumulates at about 4 days after the onset of germination, being maintained in high concentrations in the cotyledons during approximately 10 days before being degraded. This pattern of accumulation coincides

with the most critical and sensitive phase of the plant development to predation and harsh environmental conditions. Indeed, during the first 4 days of germination, the seedlings are still below the ground and thus naturally protected from most environmental stresses.

The genes encoding many antifungal proteins are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field (Lamberth *et al.,* 2013). The natural susceptibility of proteins to denaturation that results from the typical low free energy required to stabilize mature proteins hampers the direct application of antifungal proteins as fungicide sprays over crop foliage. The exploitation of antifungal proteins extracellularly but in planta or extra planta would require polymers with a reinforced tertiary structure.

From a technological point of view, BCO displays a strong antifungal activity, which confers great potential as an antifungal compound for field applications. Its inherent and wide spectrum antifungal activity, initially demonstrated under *in vitro*, is responsible for BCO equal or better performance on the control of strawberry *B. cinerea* and grapevine *E. necator* pathogens under real, open air agricultural conditions, than the best commercial, top chemical fungicides available worldwide. In addition, its extreme resistance to denaturation may allow its use under field conditions; the high susceptibility to proteolytic attack and the specificity of its biological activities probably makes it harmless to the environment and nontoxic to man and animals.

In recent years the decline and disappearance of bee species in the wild and the collapse of honey bee colonies have concerned ecologists and apiculturists, who search for causes and solutions to this problem. Whilst biological factors such as viral diseases, mite and parasite infections are undoubtedly involved, it is also evident that pesticides applied to agricultural crops have a negative impact on bees (Sanchez-Bayo and Goka, 2014). LD_{50} results obtained for honeybee toxicity studies clearly points that BCO is non-toxic for honeybees being this an imperative requisite for a new active ingredient. BCO can be in the future an important alternative for the agriculture around the world.

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2.5 References

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Chapter 3

Blad-Containing Oligomer fungicidal activity on human pathogenic yeasts. From the outside to the inside of the target cell

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Chapter 3 - Blad-containing oligomer fungicidal activity on human pathogenic yeasts. From the outside to the inside of the target cell

Abstract

Blad polypeptide comprises residues 109 to 281 of *Lupinus albus* β-conglutin precursor. It occurs naturally as a major subunit of an edible, 210 kDa oligomer which accumulates to high levels, exclusively in the cotyledons of *Lupinus* seedlings between the 4th and 14th day after the onset of germination. Blad-containing oligomer (BCO) exhibits a potent and broad spectrum fungicide activity towards plant pathogens and is now on sale in the US under the tradename Fracture™. In this work we demonstrate its antifungal activity towards human pathogens and provide some insights on its mode of action. BCO bioactivity was evaluated in eight yeast species and compared to that of amphotericin B. BCO behaved similarly to amphotericin B in what concerns both cellular inhibition and cellular death. As a lectin, BCO binds strongly to chitin. In addition, BCO is known to possess 'exochitinase' and 'endochitosanase' activities. However, no clear disruption was visualized at the cell wall after exposure to a lethal BCO concentration, except in cell buds. Immunofluorescent and immunogold labeling clearly indicate that BCO enters the cell, and membrane destabilization was also demonstrated. The absence of haemolytic activity, its biological origin, and its extraordinary antifungal activity are the major outcomes of this work, and provide a solid background for a future application as a new antifungal therapeutic drug. Furthermore, its predictable multisite mode of action suggests a low risk of inducing resistance mechanisms, which are now a major problem with other currently available antifungal drugs.

Keywords: *Candida albicans*. antifungal, natural product, pathogenic yeast, protein-based

3.1 Introduction

Fungal infections have become an important factor of morbidity and mortality and represent an increasing burden on medical systems (Del Poeta, 2010; Huffnagle and Noverr, 2013), being associated with unavoidable high mortality rates, similar to those caused by tuberculosis or malaria (Brown *et al.,* 2012).

Candida species are regarded as common components of the body microbiota in healthy humans (Kathiravan *et al.,* 2012) but are also responsible for candidaemia, an invasive fungal infection associated with substantial morbidity, mortality and healthcare costs (Zaoutis *et al.*, 2005; Bassetti *et al.,* 2015), being among the top ten pathogens causing bloodstream infections. Although *C. albicans* still remains the most abundant and significant species associated with the disease, other medically important species of *Candida* are rising (Klepser 2011; Papon *et al.,* 2013; Sardi *et al*., 2013; Won *et al*., 2015) including *C. glabrata, C. rugosa, C. parapsilosis, C. tropicalis, C. dubliniensis, C. krusei*, and *C. lusitaniae* (Kathiravan *et al.,* 2012; Huffnagle and Noverr, 2013; León *et al.,* 2014). A number of factors may explain this gradual change in epidemiology, such as severe immunosuppression or illness, prematurity, exposure to broad-spectrum antibiotics and older patients (Sardi *et al.,* 2013).

Some microorganisms are naturally resistant to certain types of antifungal medications while other species, although susceptible to a particular type of medication, have been registered as developing resistance over time as a result of improper antifungal use (Lortholary *et al.,* 2011; Shah *et al.,* 2012). Therefore, new formulations of antifungals, combination therapies and development of new bioactive compounds may be the key for a better therapeutic outcome (Spampinato and Leonardi, 2013), especially considering that antifungal research is stagnant, when compared to other pathologies. In the last decades, just a few new antifungal agents were unveiled and they were mainly based on the structural modification of already discovered drugs (Rubbiani *et al.*, 2016).

Conventional therapies against fungi rely on a very limited number of drugs (Myung and Klittich, 2015; Rubbiani *et al.*, 2016), from four different classes, and their antifungal activity and mode of action is well reviewed in the literature: polyenes (Patterson 2006; Palacios *et al.,* 2007; Denning and Hope, 2010; Cornely *et al.,* 2012; Mesa-Arango *et al.,* 2014; Nett and Andes, 2016), azoles (Odds *et al.,* 2003; Chapman *et al.,* 2008; Cui *et al.,* 2015), echinocandins (Spampinato and Leonardi, 2013; Cui *et al.,* 2015), and pyrimidine analogs (Spampinato and

Leonardi, 2013; Nett and Andes, 2016; Prasad *et al*., 2016). Despite the introduction of new antifungal agents, the clinical outcomes for most invasive fungal infections are far from ideal (Roemer and Krysan, 2014). It is therefore imperative to continue the search for different strategies to combat fungal infections (Spampinato and Leonardi, 2013; Garrigues *et al.,* 2016; Prasad *et al.*, 2016).

Antimicrobial peptides and proteins are produced by multicellular organisms as a defense mechanism against competing pathogenic microbes (Selitrennikoff, 2001) and have been considered as candidates for the development of novel antimicrobial compounds (Fjell *et al*., 2012; Virágh *et al.,* 2015; Garrigues *et al.,* 2016). A main hurdle that has hindered the development of both natural and non-natural antimicrobial peptides and proteins as therapeutic agents is the fact that many of them exhibit antifungal activity *in vitro* (e.g. magainin), but are only effective *in vivo* at very high, often toxic, levels (Darveau *et al*., 1991; Zasloff, 2002). An additional difficulty associated to the potential use of proteins is their inherent typical instability. Currently there are only five antifungal peptides recorded as having reached the clinical stage of the drug development cycle (Duncan and O'Neil, 2013). The most prominent group within the antifungal peptides are the defensins from plants, insects and mammals (Hegedüs and Marx, 2013). Plant defensins, like Psd1 (Lobo *et al*., 2007), Nad1 (Van Der Weerden *et al*., 2008) and MtDef4 (Sagaram *et al*., 2013) specifically interact with fungal membrane sphingolipids and phospholipids, enter the cell and interfere with nuclear and cytosolic proteins (Vriens *et al*., 2016).

A remarkable, novel antifungal 20.4 kDa polypeptide was recently described. It is the major subunit of a 210 kDa glyco-oligomer, termed Blad-containing oligomer (BCO), which accumulates abundantly in *Lupinus albus* cotyledons between days 4 and 12 after the onset of germination. The BCO appears to reunite in a single molecule several selected characteristics, making it a versatile, multifunctional protein (Monteiro *et al*., 2010). Its extreme resistance to chemical inactivation but high susceptibility to proteolytic attack (Monteiro *et al*., 2015), associated to a powerful and broad spectrum antifungal activity towards plant pathogens makes it a unique, flexible and environmental friend active ingredient, now on sale in the US under the tradename Fracture™.

In this work we demonstrate that BCO also has a higher inhibition potency for human pathogens than the azoles and similar to amphotericin B (AMB) on a molar basis (Monteiro *et al*., 2015), making the BCO a very promising clinical antifungal agent. We also provide some insights on its highly complex and multitarget mechanism of action.

3.2 Materials and methods

3.2.1 Microorganisms. Eight yeast strains were used, six belonging to *Candida* spp., one *Cryptococcus neoformans* strain (CBS 132) and one *Saccharomyces cerevisiae* strain (W303). *Candida* strains used were *C. albicans* var. *albicans* (CBS 562), *C. dubliniensis*, *C. glabrata*, *C. lusitaneae, C. parapsilosis* (PYCC 2597) and *C. tropicalis*. [CBS – Centraalbureau voor Schimmelcultures; PYCC – Portuguese Yeast Culture Collection; The other strains were a kind gift of Institute of Microbiology, Faculty of Medicine of the University of Coimbra (Paulo *et al*., 2009)]. All yeasts were grown at 35 °C for 24 h, except for *C. neoformans* that was grown for 72 h, in Glucose Yeast Peptone (GYP) medium (0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar). For the different experiments performed, three media were used: RPMI 1640 (Applichem), pH 7.0, supplemented with 2.08% (w/v) glucose and 6.9% (w/v) MOPS [3-(*N*-morpholino)propanesulfonic acid]; YNB (Difco), pH 7.0, supplemented with 2% (w/v) glucose and 0.1% (w/v) MOPS; and PDB (DIFCO), buffered at pH 7.5.

3.2.2 Lupinus albus and BCO purification. Dry seeds of *L. albus* were germinated and grown in growth chambers with a photoperiod of 16 h light/8 h dark at 18 °C, for periods up to 10 days. The seed coats were removed and the intact cotyledons were dissected from the axes and stored frozen at -80 °C until needed. BCO is a breakdown product of β-conglutin catabolism, and it was extracted and isolated from the cotyledons of eight-days old seedlings as described by (Monteiro *et al*., 2015). The protein corresponding to β-conglutin was purified by AKTA anion exchange chromatography followed by AKTA gel filtration chromatography as follows: the total globulin fraction was loaded on the Q-Sepharose column (Ø =1 cm; h= 8 cm; flow rate =1.5 mL/min) previously equilibrated in 20 mM Tris-HCl buffer, pH 7.5, and eluted with a linear gradient of NaCl (0 to 1 M). The fraction containing the BCO, eluted between 0.25 and 0.35 M NaCl and was subsequently subjected to gel filtration on an AKTA Superose 12 HR 10/30 column (GE Healthcare Life Sciences), equilibrated in 0.1 M Tris-HCl buffer (pH 7.5). This last purification step does not affect the polypeptide pattern of the protein, but removes unidentified low molecular mass compounds, resulting in a high pure BCO sample.

3.2.3 Antifungal agents. The BCO was extracted and purified as described above and stored lyophilized at room temperature. Amphotericin B (AMB) was obtained from their respective manufacturers and stock solutions were prepared and stored frozen at -20 °C until used.

3.2.4 Production of BCO polyclonal antibodies. A sample of BCO was lyophilized and resuspended in Freund adjuvant. New Zeland female rabbits and rats were immunized with the purified BCO sample. To obtain a high titer, three boosters injections of 100 μg/mL of antigen each were given every 2 weeks in complete Freund's diluted 1:10 with incomplete adjuvant. Total blood was taken from the heart 12 days after the third booster injection. Blood samples were allowed to clot, and the serum was collected, centrifuged, and stored at -70°C. To purify the IgG present in the serum, a chromatography in a Protein G sepharose column was conducted.

3.2.5 Antifungal susceptibility tests. Susceptibility tests were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guideline M27-A2 (NCCLS. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition. NCCLS document M27-A2 [ISBN 1-56238-469-4]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.) with some adjustments, using the broth microdilution method. Yeast cells were grown on GYP medium and the inoculum suspension was prepared by picking fresh colonies and resuspending them in 5 mL of sterile 0.9% (w/v) saline (NaCl). The resulting suspension was vortexed for 15 s and the cell density was adjusted with a spectrophotometer to give an inoculum concentration of 10^6 cells per mL. The final inoculum suspension was prepared by a 1:50 dilution followed by a 1:20 dilution with double-strength broth medium, which resulted in a final concentration of 10^3 cells per mL. One other final inoculum concentration was tested in *C. albicans*, 10^5 cells/mL, achieved by a 1:10 dilution with double-strength broth medium, for allowing a sufficient number of cells to be visualized under the microscope and to enable the determination of a MFC based on a 99.9% killing (see Minimum Fungicidal Concentration section below). The inoculum size was verified by enumeration of CFUs obtained by subculturing on GYP plates. The solution of the BCO was prepared in ultrapure sterile water and 200 μL were added to the first line of the microplate. A serial two-fold dilution was made, twelve times, using ultrapure sterile water, in the 96-well microplates. The final concentration of the BCO, after addition of the inocula, ranged from 0.002 to 4.762 μM when using PDB medium and from 0.012 to 23.81 μM with RPMI medium. The serial two-fold dilutions of AMB ranged from 0.03 to 17.31 μM. The yeast inoculum (100 μL) was added to each well of the microplate, containing 100 μL of the drug solution (two-fold concentrated). The final volume in each well was 200 μL. The microplate was incubated at 35

°C and examined after 72 h. Minimum inhibitory concentrations (MICs) are the lowest drug concentration showing absence of growth, as recorded visually. To evaluate the effect of sorbitol on the fungal susceptibility to BCO, the growth medium (two-fold concentrated) was supplemented with 2.4 M sorbitol (final concentration 1.2 M). All these tests were performed with three different batches of the BCO (triplicates).

3.2.6 Minimum fungicidal concentrations (MFCs). MFCs were determined according to (Espinel-Ingroff, 1998). After MIC determination, as previously described, 30 µL aliquots were subcultured from each well that showed no visual growth onto GYP plates. This procedure was performed to minimize drug carryover effects (Espinel-Ingroff, 1998). The plates were incubated at 35 °C for 24 h. All these tests were performed with three different batches of BCO (triplicates).

The non-existence of a standard method for determining MFCs in yeasts as led to an indiscriminate use of a wide range of methodologies for determining this value (Espinel-ingroff, 2001; Johnson *et al*., 1998; Vazquez *et al*., 1997). To maintain the standardized methodology for determining MIC, an initial inoculum of 10^3 CFU/mL was used, although it does not allow the detection of 99.9% killing. To minimize this restriction, the MFC considered in this study was the lowest drug concentration where no growth was observed after plating 30 µL on GYP plates (0 CFUs). For achieving a 99.9% killing an initial inoculum size of 10^5 CFU/mL was tested for *C. albicans* only.

3.2.7 Time-kill curves. The effect of the BCO on the growth of human fungal pathogens was evaluated by using *C. albicans* as a model organism and by comparing the results observed with those of AMB, in PDB pH 7.5. The assays were conducted in the presence of different BCO and AMB concentrations. A cell suspension was grown overnight in 20 mL of PDB pH 7.5, at 35 °C, 150 rpm and refreshed in 20 mL of PDB pH 7.5, approximately 5 h before addition to the culture medium. To obtain an initial concentration of approximately 10^5 CFU/mL, the $OD_{640\ nm}$ was adjusted to 0.1 and then 10-fold diluted with PDB pH 7.5, to a final volume of 100 mL, in 500 mL Erlenmeyer flaks. The cultures were incubated at 35 °C without shaking. At regular intervals, samples were collected for absorbance measurements, viable cell counts and morphological evaluation. For viable cell counts, 30 µL aliquots of the culture were taken, diluted if needed, and plated on GYP agar plates. Each time-kill curve was performed in triplicate and a representative curve is shown in the results.

3.2.8 Effect of BCO on yeast cell volume. The effect of the BCO on the yeast cell volume was evaluated using *S. cerevisiae* (W303) as a yeast model. Two yeast cultures were grown as described in the Time-kill curves section, one in the presence of the BCO and the other kept as control. At regular intervals, samples were collected and an estimate of the cell volume was made, by measuring the diameter of 100 cells. The cell volume was calculated considering the shape of the cells as a sphere. At each sampling time the average volume of the 100 cells was calculated as well as the corresponding standard deviation.

3.2.9 Haemolytic activity. The haemolytic activity of the BCO was analyzed according to (Ling *et al*., 2015). Briefly, fresh red blood cells from rabbit were collected and washed with PBS until the upper phase was clear after centrifugation. The pellet was resuspended in PBS to an $OD_{600\ nm}$=24 and added to a 96-well microplate. The solution of the BCO was prepared in ultrapure sterile water and a serial two-fold dilution was made in water and added to the wells. The final concentration of the BCO ranged from 0.04 to 4.76 µM. After 1 h incubation at 37 °C, cells were centrifuged at 1000 *g* and the supernatant was diluted and measured at 450 nm in a BioTek's Take3™ Multi-Volume Plate spectrophotometer.

3.2.10 Viability assessments. The LIVE/DEAD® Yeast Viability Kit (Molecular Probes) was used to evaluate fungal viability. A FUN1 100 µM working solution was prepared in 10 mM MOPS buffer, pH 7.2, with 2% (w/v) glucose. A 50 µM calcofluor white working solution was prepared in distilled water. Forty µL of fungal culture and 5 µL of FUN1 working solution were mixed thoroughly and incubated at 30 °C in the dark. After 30 min, 5 µL of calcofluor white working solution were added to the culture and mixed thoroughly. Five µL of the cell culture were trapped between a microscope slide and a coverslip for visualization on a fluorescence microscope.

3.2.11 Cell membrane integrity. After 24 h incubation with the BCO, under the same conditions as described in the Time-kill curves section, cells were incubated with propidium iodide at a final concentration of 7.5 µM, for 10 min at 4 °C. Five µL of the cell culture were trapped between a microscope slide and a coverslip, for visualization on a fluorescence microscope.

3.2.12 Morphological changes in C. albicans cells in the presence of BCO. Morphological changes in *C. albicans* cells in the presence of the BCO were assessed by Transmission Electron Microscopy (TEM). The culture was prepared and kept under the same set of conditions as previously described in the Time-kill curves section. At regular intervals, samples were collected, washed twice with saline (9.5% (w/v) NaCl) and concentrated by centrifugation (3500 *g* for 10 min) to a final concentration of 1 to $5x10^6$ CFU/mL. Cell concentration was confirmed at all points by plating in GYP agar. The samples were collected and fixed in 2% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4. Then, they were post fixed in 2% (w/v) OsO_4, dehydrated and embedded in epon. The ultrathin-sections (60 nm) were counterstained with aqueous uranyl acetate solution and lead citrate. Controls were prepared under the same conditions, but in the absence of BCO.

3.2.13 Immunolocalization of BCO. Immunolocalization of BCO was accomplished by both indirect immunofluorescence and immunogold methods. In both cases the culture was prepared and kept under the same conditions as previously described in the Time-kill curves section.

Immunofluorescency studies were accomplished according to (Lawrence *et al*., 2004) with some modifications. After 24 h incubation with a lethal concentration of the BCO, the culture was concentrated by centrifugation (3500 *g* for 10 min) to a final concentration of 1 to $5x10^7$ CFU/mL followed by treatment with lyticase (0.4 mg/mL in 500 mM Tris-HCl, 1 M sorbitol, 0.8 M KCl, 10 mM $MgS0_4$, pH 7.5) for 2 h at 30 °C, to digest the cell wall, allowing the subsequent cell membrane permeabilization. The culture was washed with PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 2.7 mM KCl) followed by fixation in 4% (v/v) formaldehyde, for 30 min, at 30 °C. After two washes with PBS and PBS containing 0.1% (v/v) Triton X-100 for cell membrane permeabilization, cells were blocked with bovine serum albumine (BSA) 5% (w/v) in PBS containing 0.1% (v/v) Triton X-100 for 30 min. Cells were washed with PBS and incubated with the first antibody (anti-BCO), produced in rabbit and diluted 1:500 in PBS containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) BSA, for 16 h at 4 °C. The cells were then washed in PBS and incubated with a second, anti-rabbit antibody, produced in goat, conjugated with FITC and diluted 1:80 in PBS with 1% (w/v) BSA, for 1 h at 37 °C. After washing twice with PBS for 15 min, 50 µM calcofluor white were added and 5 µL of the cell culture were trapped between a microscope slide and a coverslip, for visualization on a confocal microscope.

For immunogold analysis, the samples were collected after 6, 12 and 24 h of incubation with the BCO and subsequently fixed in 0.1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 1 h. Then, they were dehydrated and embedded in LRWhite. Thin sections on TBS were immunolabeled after incubation on etching process: grids were incubated in a humid chamber in large drops of a saturated aqueous solution of sodium metaperiodate, for 1 h at room temperature (RT). After washing the grids, they were first incubated for 20 min in 2% (w/v) gelatin in TBS, and a second 5 min incubation was done with 15 mM glycine. The grids were blocked with a solution containing 2% (w/v) immunoglobulin-free BSA. Sections were then incubated overnight (16 to 18 h) with the first antibody (anti-BCO) diluted 1:250 in TBS containing 2% (w/v) BSA and 1% (v/v) Tween-20 / 3% (w/v) NaCl. The grids were then washed by floating them on drops of 0.1% (w/v) BSA /TBS (four changes, 2 min each) followed by 20 min incubation on TBS with 1% (w/v) BSA. Bound antibodies were visualized by incubating the sections for 1 h with the second antibody-gold conjugate (10 nm diameter particles) diluted 1:25 in PBS with 1% (w/v) BSA, 1% (v/v) TBS. Finally, grids were washed on drops of water (six changes, 10 min each). The immune-complexes formed were visible as little black dots when observed by TEM.

3.2.14 Microscopy. *I) Fluorescence microscopy*. Samples were observed under a fluorescence microscope (Axioscope A1 with phase contrast and epi-fluorescence, Zeiss) equipped with a camera (AxioCam ICm1, Zeiss), using three different filters: Filter Set 49 DAPI (Excitation G 365, Emission BP 420/470); Filter Set 10 FITC/GFP (Excitation BP 450-490, Emission BP 515-565) and Filter Set 15 Rodhamine (Excitation BP 540-552, Emission LP 590). *II) Confocal microscopy*. The images were acquired with a Leica TCS SP5 II confocal microscope equipped with an objective HCX PL APO CS 63x/1.3 Glycerol (Leica Microsystems, Germany). Samples were excited by 488 nm laser line and emitted signal detected in the range of 496-564 nm with a HyDet detector (fluorescence channel) and a PMT detector (transmission channel). Images were acquired with 512 x 512 pixels and a pixel size of 68 nm. *III) Transmission electron microscopy*. The sections were examined under a JEOL JEM 1400 TEM 120kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan.

3.3 Results

3.3.1 Determination of minimum inhibitory and fungicidal concentrations. The antifungal activity of three different batches of BCO was evaluated in six strains belonging to six *Candida* species and in one strain of *C. neoformans,* with each strain cultured in two different growth media*. C. albicans* CBS 562 was also tested for resistance to AMB, one commonly used antifungal drug. The MICs and MFCs of BCO are shown in Table 3.1. MIC values in RPMI medium for the entire group varied from 0.19 to 2.98 μM. Regarding the MFCs of BCO, the values obtained were higher than the highest concentration tested. The exception was the strain of *C. albicans*, for which the MFC of BCO was 23.81 μM (Table 3.1).

When the antifungal activity of BCO was assessed in PDB medium, the MIC and MFC values obtained were more consistent among the different species, which is in accordance with previous studies that demonstrated that PDB is a more suitable medium for testing the bioactivity of this oligomer (Monteiro *et al*., 2015). All strains tested presented a similar susceptibility to BCO, with MIC values ranging from 0.08 to 0.31 μM (Table 3.1). Regarding the MFCs of BCO in PDB medium, the values varied from 0.15 to 2.38 μM. For the strains of *C. albicans*, *C. dubliniensis* and *C. neoformans*, the MFCs were always only twice of the respective MICs and for those of *C. glabrata* and *C. lusitaneae* were always 4 times higher. The MFC of BCO for *C. parapsilosis* was too much higher than the other ones (2.38 μM) (Table 3.1). Overall the results obtained for BCO are indicative of a potent antifungal activity for these fungal species.

The antifungal activity of BCO was then compared to that exhibited by AMB, in PDB medium, using *C. albicans* as a control model. This specie has long been used as a model in several fungal research studies (Fu *et al*., 2008; Kabir *et al*., 2012), and also showed a strong susceptibility to BCO, as demonstrated by the corresponding MIC and MFC values (Table 3.1). Since the optimum inoculum density for the subsequent microscopy tests was found to be 10^5 CFU/mL, and since this concentration allows the detection of a 99.9% killing based MFC, both minimum inhibitory and fungicidal concentrations were determined with this inoculum size. The results are presented in Table 3.2. As expected, there was an increase in MIC and MFC values, due to a higher number of cells in the initial inoculum. However, these results are still consistent with those obtained before (Table 3.1). The concentration of BCO needed to induce death of 99.9% of the microorganisms was only twice the minimum inhibitory concentration (0.60-1.19 and 1.19-2.38 μM, respectively). The same effect was observed for AMB (MIC and MFC values of 1.1

and 2.2 μM, respectively), which is also consistent with the values reported in the literature (Manavathu *et al*., 1998; Spreghini *et al*., 2012).

Table 3.1. Ranges of MIC and MFC values of BCO for various yeast species grown in two different culture media, with an initial inoculum of 10^3 CFU/mL and tested with three different batches of BCO.

	RPMI medium		PDB medium	
Yeast Species	**MIC (μM)**	**MFC (μM)**	**MIC (μM)**	**MFC (μM)**
Candida albicans	1.49	23.81	0.15-0.31	0.31-0.61
Candida dubliniensis	0.75-1.49	>23.81	0.15-0.31	0.31-0.61
Candida glabrata	0.19-0.37	> 23.81	0.08-0.15	0.31-0.61
Candida lusitaneae	0.37	> 23.81	0.15-0.31	0.61
Candida parapsilosis	0.37	> 23.81	0.15-0.31	2.38
Candida tropicalis	2.98	> 23.81	0.15	0.61
Cryptococcus neoformans	0.37	>23.81	0.08-0.15	0.15-0.31

Table 3.2. Ranges of MIC and MFC values of BCO and AMB for *C. albicans* (in PDB medium at pH 7.5), with an initial inoculum of 10^5 CFU/mL and tested with three different batches of BCO.

Antifungal agent	MIC (μM)	MFC (μM)
BCO	0.60-1.19	1.19-2.38
AMB	1.1	2.2

3.3.2 BCO leads to an increase on the yeast cell volume unrelated to major cell wall disturbances. Given the demonstrated affinity of BCO to chitin (Monteiro *et al*., 2015), two studies were conducted for assessing its possible effect in cell wall biosynthesis. *S. cerevisiae* was used as the model organism for these studies. The cells were exposed to BCO at the MIC value and an estimate of the cell volume at three-time sampling points was made. The results obtained are described in Figure 3.1 and show that the presence of BCO leads to a progressive increase in the average volume of the cells. The volume variation between the first and the last sample analyzed is higher than 10 fold (147 μm^3 after 1 h of incubation and 1621 μm^3 after 48 h).

Considering the results above, if BCO's mode of action is at least in part based on cell wall damage, it is expected for the cell to suffer an increase of volume under hypotonic conditions, until eventually bursts. If this is the case, under isotonic conditions, or even in a slightly hypertonic medium, this effect should not occur, since there is no water entering into the cells. With the purpose of testing if the osmotic stabilizer sorbitol counteracts the toxicity of BCO, 1.2

M sorbitol was added to the culture medium (creating a slightly hypertonic condition). Tests were carried out in the absence and in the presence of 1.2 M sorbitol in *S. cerevisiae.* The results demonstrated that the presence of 1.2 M sorbitol in the culture medium did not reduce the antifungal effect of BCO, since the MIC values were the same in both cases (0.15 µM). This result suggests that ultimately, the toxicity of BCO to fungi is not dependent on dramatic changes in cell wall integrity despite its ability to bind very tightly to the chitin polymer (Monteiro *et al*., 2015).

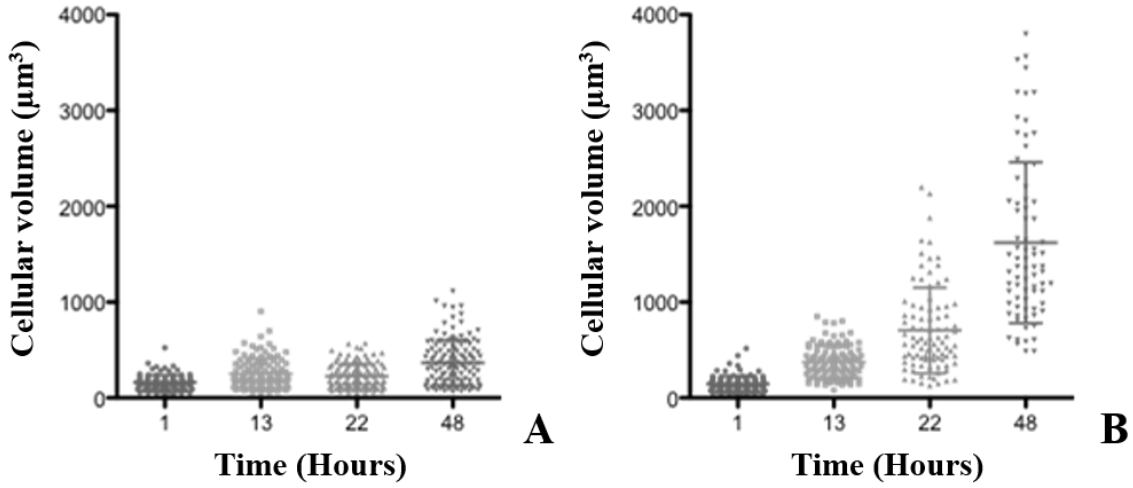

Figure 3.1. BCO effect on S. cerevisiae W303 cell volume along time. S. cerevisiae cells were grown in YNB medium supplemented with 2% (w/v) glucose, pH 7.0, at 30 °C. The horizontal bars show the mean values (central bar) and the standard deviation (edge bars). (**A**) – culture kept without the BCO; (**B**) – culture with 0.152 µM BCO.

3.3.3 BCO has a dose-dependent effect on the growth of *C. albicans*. Determination of the "killing" of an isolate over time by one or more antimicrobial agents under controlled conditions is known as the time-kill method (Pfaller *et al*., 2004). It is a broth based method where the rate of killing of a fixed inoculum is determined by sampling control (organism, no drug) and antimicrobial agent-containing tubes or flasks, at certain time intervals, and determining the survivor colony count (CFU/mL) by spreading each sample onto an agar plate. In order to study the effect of BCO on the growth of *C. albicans*, time-kill curves were performed in PDB medium. Several samples were taken during these experiments in order to assess the evolution of the number of viable cells ($OD_{640\ nm}$ and CFU counts). Two concentrations of BCO were used, 1.19 µM and 2.38 µM, corresponding to the minimum inhibitory and fungicidal

concentrations, respectively, as determined previously (Table 3.2). A fraction of the culture grown under the same conditions but without BCO was tested for control purposes. The results are shown in Figure 3.2 for BCO and in Figure 3.3 for AMB.

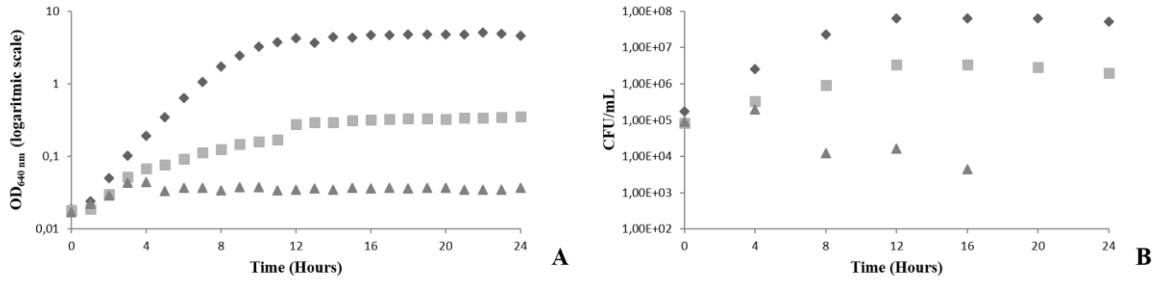

Figure 3.2. Effect of BCO on the growth of *C. albicans* in PDB medium, pH 7.5, 35 °C, without agitation (representative curve of triplicate experiments). **A** – $OD_{640\ nm}$ **B** – CFU/mL. BCO concentration in the culture medium: 0 µM (♦), 1.19 µM (■) and 2.38 µM (▲).

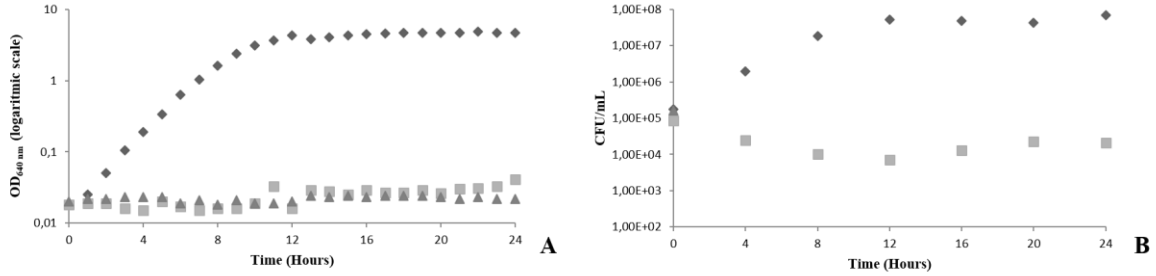

Figure 3.3. Effect of AMB on the growth of *C. albicans* in PDB medium, pH 7.5, 35 °C, without agitation (representative curve of triplicate experiments). **A** – $OD_{640\ nm}$ **B** – CFU/mL. AMB concentration in the culture medium: 0 µM (♦), 1.1 µM (■) and 2.2 µM (▲).

Figure 3.2 shows that the addition of BCO to the culture medium had a strong effect in the growth of *C. albicans.* It is possible to observe that the culture grown in the absence of BCO presents a normal growth curve, being in the exponential phase of growth for approximately 12 h, before entering the stationary phase. This was observed by following both $OD_{640\ nm}$ readings and CFU/mL counts. Cells grown in the presence of the minimum inhibitory concentration of BCO, showed a decrease in the growth rate when compared to the control, which resulted in a lower final optical density (Fig. 3.2A) and a lower final CFU/mL count (Fig. 3.2B). This result indicates that the concentration of BCO tested had, indeed, the ability to inhibit the growth of this microorganism. Cells grown in the presence of the minimum fungicidal concentration became non-viable after 16 h of growth. This was observed by both

stabilization of $OD_{640\ nm,}$ just after 4 hours of incubation (Fig. 3.2A), after a slight initial growth, and absence of CFU counts (Fig. 3.2B).

The same assay was performed using AMB as the antifungal agent. Two different concentrations were also used, 1.1 μM and 2.2 μM, corresponding to the minimum inhibitory and fungicidal concentration, respectively. The results obtained were very similar to those obtained for the BCO and are shown in Figure 3.3. The control fraction stayed in exponential phase for 12 h, and the fraction exposed to the minimum inhibitory concentration showed a total absence of growth. When using the minimum fungicidal concentration of AMB cells became non-viable in the first 4 h of exposure, which was observed by the absence of CFU counts (Fig. 3.3B). The prompt reduction in *C. albicans* viability caused by AMB is in accordance with the data published in the literature (Leite *et al*., 2014; Cantón *et al*., 2004). The major difference between the BCO and AMB in terms of killing kinetics is that both fungistatic and fungicidal activities of AMB, under these conditions, act more rapidly than those of the oligomer.

3.3.4 The MFC of BCO induces a severe decrease on the metabolic activity in *C. albicans*.

The effect of BCO on the viability and cellular integrity of yeasts was evaluated using *C. albicans* as model and was assessed using samples collected along the growth curve, in PDB medium, under three different conditions: without drug (control), with the inhibitory (MIC) concentration (1.19 μM) and with the lethal (MFC) concentration (2.38 μM). Each sample was stained with FUN-1 and calcofluor white and visualized in a fluorescence microscope. FUN-1 binds to nucleic acids producing a yellowish green fluorescence in death cells with a damaged membrane. Cells without metabolic activity but with an intact plasma membrane also present a diffuse green coloration in the cytoplasm. On the other hand, in metabolically active cells, formation of orange cylindrical structures designated CIVS (Cylindrical IntraVacuolar Structures) is observed inside vacuoles. CIVS formation only occurs in metabolically active cells with an intact plasma membrane, meaning they are not observed in dead cells (Chew *et al*., 2015). Calcofluor white is a compound with high affinity to chitin and is normally used as a marker of the fungal cell wall. The results are shown in Figures 3.4 and 3.5.

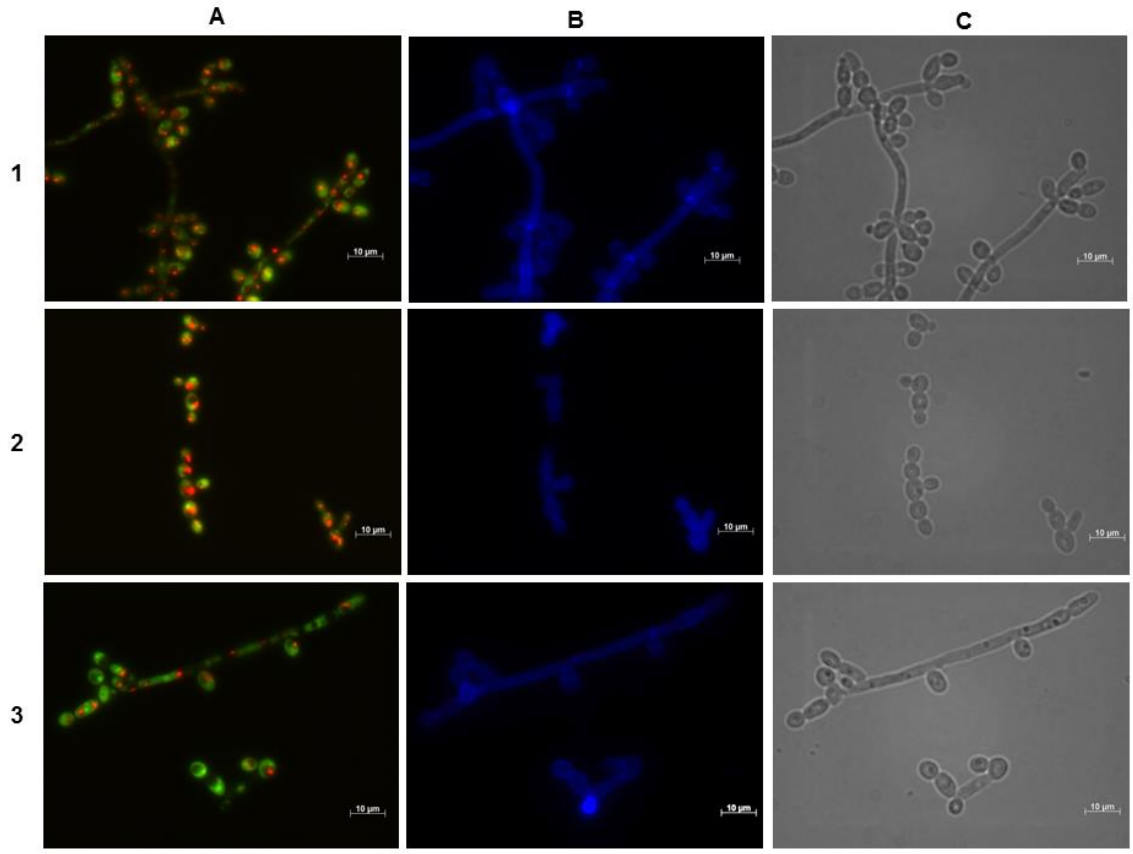

Figure 3.4. Effect of BCO on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 °C, without agitation. Samples were taken after 4 h of incubation. Concentration of BCO in the culture medium: 1 – 0 µM, 2 – 1.19 µM, 3 – 2.38 µM. Labeling with FUN-1 (**A**), calcofluor white (**B**) and bright field microscopy (**C**). Bar corresponds to 10 µm.

Figure 3.4 suggests that during the first 4 h of incubation with BCO there are no changes regarding the viability and integrity of the cells, for all conditions tested, since the presence of CIVS indicates metabolic activity and the fluorescence with calcofluor white is normal and equal to the control fraction, indicating cell wall integrity. These results are consistent with the ones obtained in the growth curves (Fig. 3.2).

After 12 h of incubation with BCO, the control fraction continued to exhibit CIVS in the majority of the cells, confirming that they were still metabolically active. The fraction incubated with an inhibitory concentration of BCO showed a slight lower number of cells with CIVS, meaning that some cells were metabolically active and, therefore viable and culturable, thus explaining the small increase in the $OD_{640\ nm}$ and CFU counts observed in the curve of Figure 3.2. At 12 h, cells incubated with the lethal concentration of BCO presented very few CIVS, corresponding to a

decrease in the metabolic activity (data not shown). This explains the decrease also observed in the CFU counts observed in Figure 3.2.

Microscopical observations performed at 16 h of incubation revealed to be a turning point in cell viability, which is in accordance to what is also observed with other antifungal drugs (Kim *et al*., 2011). Although both the control and MIC fractions of BCO showed no changes as compared to the previous time point studied, the culture incubated with a lethal concentration of BCO no longer presented visible CIVS. Only a diffuse green coloration in the cytoplasm was visible, corresponding to the absence of metabolic activity (Fig. 3.5). However, when cultivated in a free BCO medium some cells were still able to grow (Fig. 3.2B). At 24 h of incubation, the last time point studied, the control fraction presented some cells without CIVS, typical of an old culture and the MIC fraction presented even fewer metabolically active cells than in the previous time point studied (data not shown). This is in accordance with the stabilization of $OD_{640\ nm}$ and with the smaller number of culturable cells observed in the growth curves (Fig. 3.2). The results obtained with the lethal concentration of BCO were similar to those obtained after 16 h (cells without any metabolic activity), but at this point there were also no records of culturable cells (Fig. 3.2B). This means that approximately between 16 to 24 h of incubation with a lethal concentration of the BCO, *C. albicans* lost the ability to grow in a free BCO medium, and that the number of culturable cells was beneath the detection limit of the method (<10 CFU/mL). These results suggest that after 16 h of incubation with a lethal concentration of BCO, cells are metabolically inactive (Fig. 3.5), non-viable and nonculturable (Fig. 3.2). During these periods, the integrity of the cell wall remained unchanged regardless of the concentration of BCO tested, as showed by calcofluor white staining (Figs. 3.4 and 3.5).

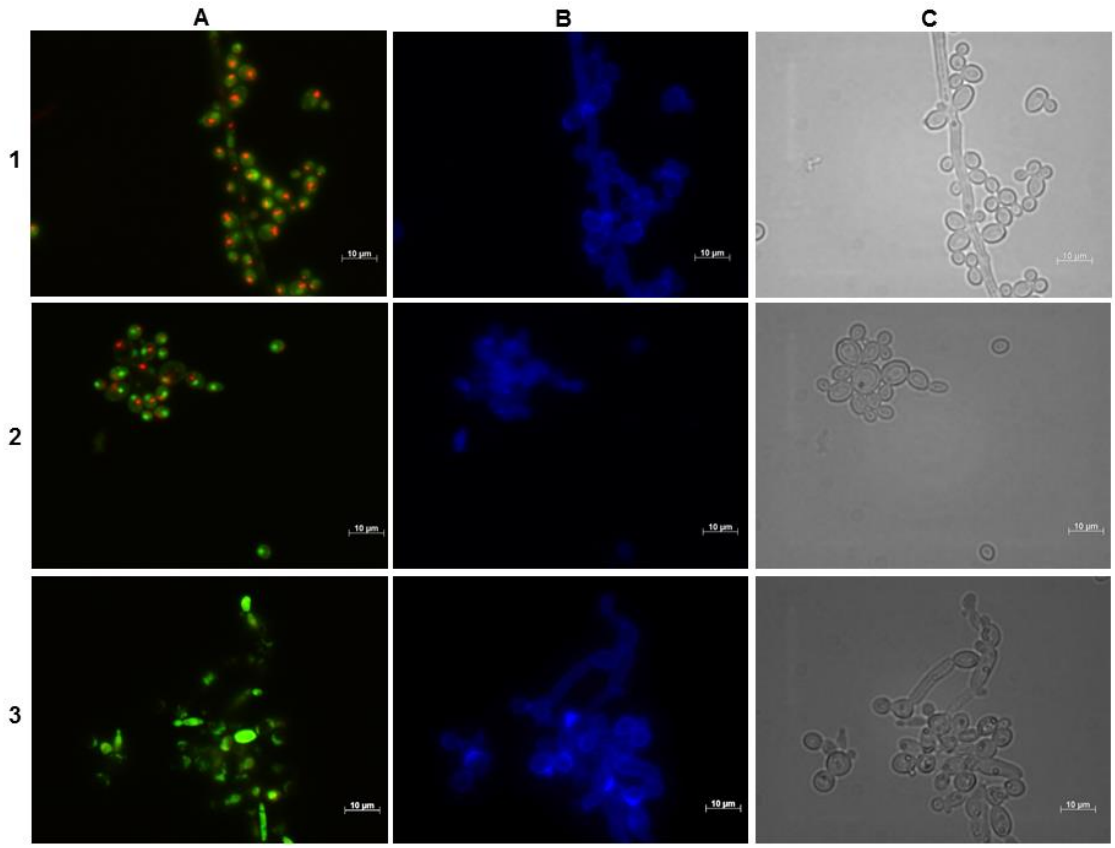

Figure 3.5. Effect of BCO on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 °C, without agitation. Samples were taken after 16 h of incubation. Concentration of BCO in the culture medium: 1 – 0 µM, 2 – 1.19 µM, 3 – 2.38 µM. Labeling with FUN-1 (**A**), calcofluor white (**B**) and bright field microscopy (**C**). Bar corresponds to 10 µm.

3.3.5 BCO induces cell membrane damages. Cell membrane integrity was evaluated with propidium iodide. This compound binds to DNA and RNA producing a red fluorescence when intercalated with nucleic acids. However, due to its positive charge, it cannot cross an intact cell membrane and, therefore, only dead cells or cells with a damaged membrane are stained. Propidium iodide staining was evaluated at 24 h of incubation of *C. albicans* with the inhibitory (MIC) and with the lethal (MFC) concentration of BCO, as determined previously (1.19 µM and 2.38 µM, respectively). The results, shown in Figure 3.6, clearly indicate that BCO somehow destabilizes the plasma membrane, enabling the entrance of the fluorescent dye into the cell.

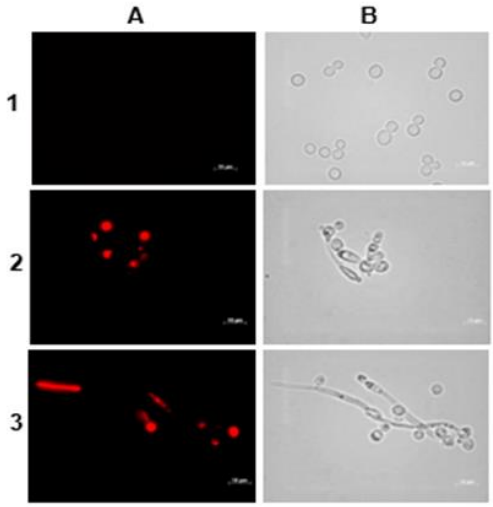

Figure 3.6. Effect of BCO on the viability and membrane integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 °C, without agitation. Samples were taken after 24 h of incubation. Concentration of BCO in the culture medium: 1 – 0 µM, 2 – 1.19 µM and 3 – 2.38 µM. Labeling with propidium iodide (**A**), and bright field microscopy (**B**). Bar corresponds to 10 µm.

3.3.6 BCO moves from the cell wall into the interior of the cell. Immunofluorescence is a technique that allows the visualization of antigen-antibody interactions in cell suspensions. To this end, BCO was used as the antigen since a first anti-BCO antibody produced in rabbit is then added, followed by a second anti-rabbit antibody produced in goat, conjugated with FITC. In this particular case, *C. albicans* was incubated with a lethal concentration of BCO (2.38 µM) in PDB pH 7.5 medium for 24 h and observed by confocal microscopy. Calcofluor white was also added to assess the efficiency of the cell wall digestion. The results are presented in Figure 3.7 and show a clear green fluorescence inside the cell. Control without BCO was also tested and no green fluorescence was observed (data not shown). This result suggests that after 24 h of incubation, BCO was able to cross the cell envelope and is clearly inside the cells. The calcofluor white staining indicates that not all the cell wall was efficiently digested (Fig. 3.7C). However, it was sufficient to allow the cell membrane permeabilization and the subsequent entrance of the antibodies.

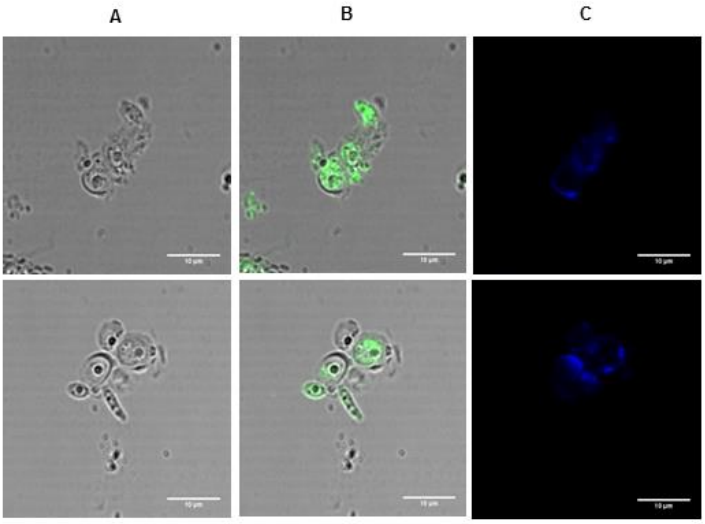

Figure 3.7. Immunofluorescence in *C. albicans* incubated with a lethal concentration of BCO for 24 h and visualized by confocal microscopy. Cells were treated with lyticase before fixation. BCO functions as the antigen; first antibody: anti-BCO produced in rabbit; second antibody: anti-rabbit produced in goat, conjugated with FITC. Bright field microscopy (**A**), bright field microscopy merged with FITC filter (**B**), DAPI filter (**C**). Bar corresponds to 10 µm.

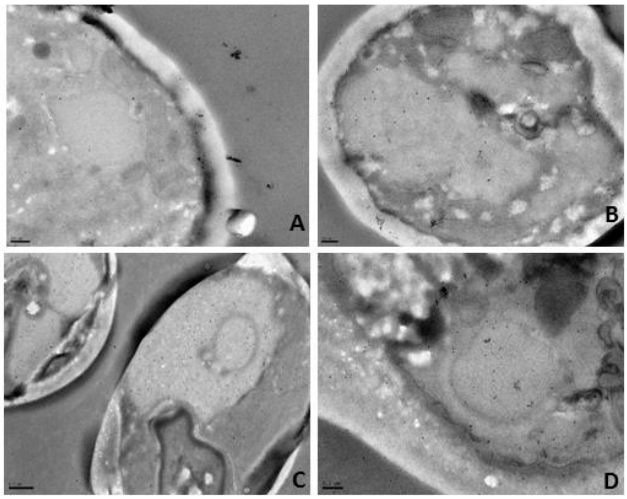

Figure 3.8. Immunogold localization in *C. albicans* after incubation with BCO for **(A)** 2 h, **(B)** 6 h, **(C)**12 h and **(D)** 24 h, as observed by TEM. The black dots represent the localization of BCO in the cells.

To confirm these results, we next proceeded with immunogold labeling. As before, cells were exposed to a lethal concentration of BCO for 24 h and samples were collected after 2, 6, 12, and 24 h of incubation. Each sample was analyzed by immunogold using anti-BCO produced in rat as the first antibody and a second anti-rat antibody coupled to gold particles. The immune-complexes formed were visible as little black dots when observed by transmission electron

microscopy (TEM) (Fig. 3.8). At each sampling point, including time zero, the controls without BCO were also tested and no black dots were found in none of the controls tested (data not shown). The analysis of Figure 3.8 shows that BCO progressively moves from the cell wall into the interior of the cell. Shorter incubation periods show the protein preferentially agglomerating near the cell wall (Figs. 3.8A and 3.8B), while longer incubation periods show a migration of BCO to the cytosol and even to the interior of vacuoles (Figs. 3.8C and 3.8D).

3.3.7 *C. albicans* suffers several morphological changes in the presence of BCO.

The morphological changes undergone by *C. albicans* after exposure to BCO, were evaluated by TEM. Cells were exposed to a lethal concentration of BCO for 48 h and samples were collected at 0, 24 and 48 h. The results are presented in Figure 3.9.

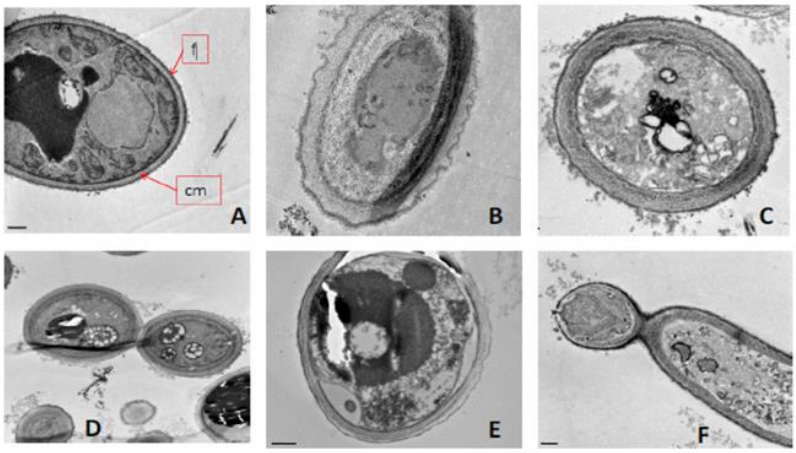

Figure 3.9. Morphological changes after exposing *C. albicans* to a lethal concentration of BCO as observed by TEM. Samples were collected and visualized at times (**A**) 0 h, (**B**) 24 h and (**C,D,E,F**) 48 h. **f** – fibrillary layer; **cm** – plasma membrane.

Figure 3.9A shows a section of a well preserved *C. albicans* cell, presenting a homogeneous cytoplasm, with an external fibrillar layer (f), a compact cell wall and a normal plasma membrane (cm). After 24 h incubation with the lethal concentration of BCO, several morphological changes are visible (Fig. 3.9B), mainly focused between the cell wall and the cell membrane. These changes became more clear after 48 h of incubation with a lethal concentration of BCO, where it is visible an increased thickness at the cell wall level (Fig. 3.9C), appearance of small vesicles in the periplasmatic region (Fig. 3.9D), abnormal density and shape of the cell wall (Fig. 3.9D), an accumulation of high density vacuoles in the cytoplasm (Fig. 3.9E) and cell wall disruption, only visible in some buds (Fig. 3.9F). Although the population of

cells showed some heterogeneity, in general, the cells that presented more structural changes also displayed an increased size. The controls performed in the absence of BCO showed none of these characteristics at the corresponding harvesting times (data not shown).

3.3.8 Absence of haemolytic activity. The interaction of BCO with mammalian red blood cells was studied by haemolysis experiments. Erythrocytes were incubated with different concentrations of BCO, ranging from 0.04 to 4.76 μM for 1 h at 37 °C. BCO showed no haemolytic effects up to 4.76 μM (data not shown), which is indicative of no detectable interference of the red blood cells.

3.4 Discussion

The data presented above clearly show that BCO has antifungal activity against a wide range of human yeasts pathogens, namely, *C. albicans, C. dubliniensis, C. glabrata, C. lusitaneae, C. parapsilosis, C. tropicalis and C. neoformans*. The antifungal activity of BCO was compared to that of AMB, a commonly used human antifungal agent, and the results demonstrate that the concentrations needed to induce both cellular inhibition and cellular death are very similar to those referenced for AMB (Cantón *et al*., 2004; Sabatelli *et al*., 2006; Cordeiro *et al*., 2013). BCO has already a sustained efficacy proved against phytopathogenic fungi, both under *in vitro* assays and in field trials (Monteiro *et al*., 2015), but this is the first report that demonstrates its activity against unicellular human pathogenic fungi.

Similarly to other antifungal compounds such as Congo red, caffeine or caspofungin, several antifungal peptides (e.g. defensins) and proteins from *Aspergillus* species are known to interfere with cell wall biosynthesis, thus weakening it by activation of the cell wall integrity pathway (CWIP) and by inhibiting chitin synthesis. Given the proven affinity of BCO to chitin (Monteiro *et al*., 2015), the presence of the osmotic stabilizer sorbitol should have resulted in a significant reduction of BCO antifungal activity if, in the end, the CWIP was its first target. However, the presence of 1.2 M sorbitol in the culture medium did not reduce the antifungal effect of BCO suggesting that ultimately, the toxicity of BCO is not directly related to dramatic changes caused in cell wall integrity.

All bioactivities described for BCO up until now, were only focused on the fungal cell wall (Monteiro *et al*., 2015). However, chitinase and β-1,3-glucanase activities were not detected in BCO, which apparently limits its ability to cleavage the cell wall of fungi. β-1,3-glucanase has been reported to partially digest the cell walls of *Verticillium albo-atrum*, and this degradation seems to be synergistically stimulated by chitinase (Skujins *et al*., 1965). Chitinases have long been reported to hydrolyse chitin from the cell walls of some fungal pathogens and nonpathogens *in vitro* (Skujins *et al*., 1965; Boller *et al*., 1983; Schlumbaum *et al*., 1986), and β-*N*-acetyl-D-glucosaminidase is only able to complete the degradation of chitin by hydrolyzing the soluble oligosaccharides to monosaccharides. β-*N*-Acetyl-D-glucosaminidase, one of the enzymatic activities displayed by BCO (Monteiro *et al*., 2015) catalyzes the progressive release of *N*-acetyl-D-glucosamine from the non-reducing end of chitin, and together with chitinases are considered a very effective chitinolytic system on fungi (Silva *et al*., 2004). For example, in

Trichoderma harzianum this system is suggested to play an important role in its potential use as a biocontrol agent against several phytopathogenic fungi (Haran *et al*., 1996). Chitosanase activity of BCO is the endohydrolysis of β-1,4 linkages between *N*-acetyl-D-glucosamine and D-glucosamine residues in partially deacetylated fungal cell wall chitosan polymer. This should not to be confused with chitinase activity, which is the random endohydrolysis of *N*-acetyl-β-D-glucosaminide β(1,4) linkages in chitin and chitodextrins. Chitosan is the deacetylated version of chitin, and may possess different degrees of deacetylation (Tsigos *et al*., 2000). Chitosan is present in the cell wall of a small number of fungi (mainly zygomycetes), conferring structural integrity (Baker *et al*., 2007). *C. albicans* cell wall, like many other fungi, does not contain chitosan, so we were not expecting to see any particular rupture in the microscopic observations with calcofluor white (a specific cell wall marker for fungi, due to its chitin affinity). However, some destabilization of the cell wall structure may occur, without any visual effect by fluorescent labeling, due to its β-*N*-acetyl-D-glucosaminidase activity, at the end of the chitin polymer. In fact, the absence of visual damages at the cell wall does not imply a fully operational and structured wall, as the same can be observed even with cell wall-targeting drugs (Piotrowski *et al*., 2015). This might explain why the cell wall remained visually intact with calcofluor white staining, while the cells were becoming metabolically inactive, or even dead. A certain degree of disturbance in the cell wall structure seems to be required to allow the entrance of the 210 kDa oligomer. Furthermore, a clear disruption of the cell wall was observed in some bud cells. Nevertheless, the antifungal activity of BCO does not seem to be related to the cell wall integrity, as demonstrated previously with the sorbitol test result.

Damages at the cell membrane level, measured by propidium iodide uptake, are usually indicators of cell death (Simonin *et al*., 2007; Choi *et al*., 2012), but it is also possible that some damaged cells retain the ability to recover after a short incubation (Davey and Hexley, 2011). Propidium iodide may also be used as an indicator of cell leakage, or cell permeability (Piotrowski *et al*., 2015). In any case, it was here demonstrated that BCO induces cell membrane damage in *C. albicans*, and this surely contributes to its inhibitory and/or lethal effect on cells. Nevertheless, and once again, this does not seem to be its primary mode of action.

Regarding the localization of BCO in the cell, the results obtained by both immunofluorescence and immunogold labeling definitely indicate that BCO enters the cell, although the mechanism underlying such entry is still unclear, especially considering its large molecular size (210 kDa). However, *C. albicans* is known to internalize large molecules similarly to other fungi (Riezman,

1985; Basrai *et al*., 1990; Helmerhorshmt *et al*., 2001; Oberparleiter *et al*., 2003; Theis *et al*., 2005), and such mechanism can explain the transport of BCO into the cytosol. There are also reports of antifungal proteins being internalized by fungal cells, like NaD1 (Van Der Weerden *et al*., 2008; Van Der Weerden *et al*., 2010), MtDef4 (Sagaram *et al*., 2013) and Psd1 (Lobo *et al*., 2007). There are several mechanisms by which these proteins can be internalized by the cell, such as receptor-mediated internalization, membrane translocation and membrane permeabilization (Vriens *et al*., 2014).

Many of the morphological changes observed in *C. albicans* cells upon exposure to a lethal concentration of BCO, namely thickening of the cell wall and an increased cell size, which are the most striking features, were already observed in yeast species as a response to various stressful conditions such as halophilic stress (Kuncic *et al*., 2010; Gao *et al*., 2014) and exposure to some antifungal drugs (Ishida *et al*., 2009; Rueda *et al*., 2014). For example, in *S. cerevisiae*, the presence of salt stress causes an abnormal cell wall structure, becoming uneven and thicker, with certain areas clearly damaged (Gao *et al*., 2014), similarly to what was observed for *C. albicans* and BCO. It is likely that the ultrastructural alterations described in this study also result from some chemical stress, at the osmotic level. Regarding BCO, some experiments demonstrate that this oligomer displays a divalent-chelating activity for several cations. This biochemical property may selectively disturb the essential divalent cation metabolism of the microorganism by interfering with metal acquisition and bioavailability for crucial reactions. This chelation activity could ultimately disturb the microbial cell homeostasis and culminate in the blockage of microbial nutrition, growth and development (Santos *et al*., 2012).

In conclusion, we demonstrate that after being exposed for more than 16 h to a lethal concentration of BCO, *C. albicans* became metabolically inactive, non viable and non culturable. Moreover, some cells showed loss of cell membrane integrity, but with no visible rupture of the cell wall, except in bud cells. The ultrastructural alterations observed suggest that BCO imposes stressful conditions upon the fungal cell, which ultimately lead to the cell death. The disturbances observed at the cell wall and membrane seem to be just a part of a more complex mode of action of BCO. In the future more studies are required in order to fully understand this complex mode of action, specifically its primary targets within the cell and the physiological mechanisms underlying cell death. The exceptional antifungal activity of BCO, combined with its natural and edible origin, and the absence of haemolytic activity, provide a solid background

to further investigate its potential as a novel antifungal therapeutic drug. Its current success as a phytopharmaceutical drug is already a good indicator to pursuit this goal. Furthermore, its predictable multisite mode of action suggests a low risk of inducing resistance mechanisms, which are now a major problem with other currently available antifungal drugs.

3.5 Acknowledgments

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Chapter 4

Bridging the gap to nontoxic fungal control: *Lupinus*-derived Blad-Containing Oligomer as a novel candidate to combat human pathogenic fungi

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Chapter 4 - Bridging the gap to nontoxic fungal control: *Lupinus*-derived Blad-Containing Oligomer as a novel candidate to combat human pathogenic fungi

Abstract

The lack of antifungal drugs with novel modes of action reaching the clinic is a serious concern. Recently a novel antifungal protein referred to as Blad-containing oligomer (BCO) has received regulatory approval as an agricultural antifungal agent. Interestingly its spectrum of antifungal activity includes human pathogens such as *Candida albicans* however its mode of action has yet to be elucidated. Here we demonstrate that BCO exerts its antifungal activity through inhibition of metal ion homeostasis which results in apoptotic cell death in *C. albicans*. HIP HOP profiling in *Saccharomyces cerevisiae* using a panel of signature strains that are characteristic for common modes of action identified hypersensitivity in yeast lacking the iron-dependent transcription factor Aft1 suggesting restricted iron uptake as a mode of action. Furthermore, global transcriptome profiling in *C. albicans* also identified disruption of metal ion homeostasis as a potential mode of action. Experiments were carried out to assess the effect of divalent metal ions on the antifungal activity of BCO revealing that BCO activity is antagonized by metal ions such as Mn^{2+}, Zn^{2+} and Fe^{2+}. The transcriptome profile also implicated sterol synthesis as a possible secondary mode of action which was subsequently confirmed in sterol synthesis assays in *C. albicans*. Animal models for toxicity showed that BCO is generally well tolerated and presents a promising safety profile as a topical applied agent. Given its potent broad spectrum antifungal activity and novel multi-target mode of action, we propose BCO as a promising new antifungal agent for the topical treatment of fungal infections.

Keywords: Blad-containing oligomer, antifungal, metal chelation, metal homeostasis, multitarget mode of action, toxicology

4.1 Introduction

Relative to antibacterial drug discovery, antifungal drug research has received less attention, (Delarze and Sanglard, 2015; Ngo *et al.,* 2016); this is despite the fact that 1.2 billion people worldwide suffer from fungal diseases (Denning and Bromley, 2015) and mortality rates may exceed those caused by tuberculosis or malaria (Brown *et al*., 2012). Unlike bacterial infections, which can be treated aggressively and with few toxic side effects, the shared eukaryotic nature of fungi and mammals presents a greater challenge when seeking selective low toxicity candidate molecules (Geiser, 2015). The pace of developing new antifungal drugs has been extremely slow, and with numerous failures (Liu *et al*., 2016). Most new promising compounds end up failing during the final development stages, mostly because of their mode of action (promoting fungal resistance) and/or toxicity issues. This low success rate has definitely discouraged the pharmaceutical industry from investing their resources on this type of research and explains the absence of new drug classes since echinocandins were introduced in 2001.

A restricted number of chemical classes are currently in clinical use as antifungal agents (Delarze and Sanglard, 2015) and novel resistance profiles to these drugs are frequently reported (Papon *et al*., 2007; Perlin, 2007; Grossman *et al*., 2015; Sanguinetti *et al*., 2015; Kołaczkowska and Kołaczkowski, 2016; Morschhäuser, 2016), including development of multi-drug resistance strains (Sanglard, 2016). Only minimal resistance to the polyene amphotericin B (AMB) has evolved despite more than 30 years of intensive clinical use (Kanafani and Perfect, 2008; Davis *et al*., 2015), probably due to its multitarget mode of action (Gray *et al*., 2012). However, AMB is well known for its side effects and toxicity (Spampinato and Leonardi, 2013; Nett and Andes, 2016). A novel class of antifungal drugs, named orotomides, is now emerging, with very promising results in dimorphic and filamentous fungi, particularly for *Aspergillus* spp., and is currently in late phase 1 clinical trials for the treatment of invasive aspergillosis (Oliver *et al*., 2016). Nonetheless, it acts via a novel mechanism of action that targets a single enzyme (inhibition of the dihydroorotate dehydrogenase), and it has no activity against human pathogenic yeasts (Oliver *et al*., 2016). Generally, drugs acting on a single cellular target are more likely to encounter the problem of drug resistance (Wong *et al*., 2014).

Recently, we reported the discovery of an edible polypeptide, named Blad, which accumulates in *Lupinus* cotyledons (Monteiro *et al*., 2015) and occurs naturally as part of a 210 kDa oligomer

(BCO, after Blad-containing oligomer). BCO exhibits potent and broad-spectrum fungicidal activity, comparing favorably to fluconazole (FLC) and AMB in *in vitro* (Pinheiro *et al*., 2016) and surpassing some commercially available fungicides in greenhouse and field conditions. BCO is currently on sale in the US, Canada and South Korea under the trade name Fracture™. Other certification processes are currently under way worldwide. The highly complex and multitarget mechanism of action of BCO (Pinheiro *et al*., 2016) was already aknowledged by the Fungicide Resistance Action Committee (FRAC), with its inclusion in a new mode of action category, M12, on the 2016 issue of the FRAC Code List© for agricultural applications.

With such a wide range of bioactivities, anyone skilled-in-the-art would expect BCO to be toxic to mammal cells as well. In this study, we demonstrate that this does not seem to be the case. In addition to its inherent capacity to bind (lectin activity) and cleave chitin (*N*-acetyl-D-glucosaminidase activity) (Monteiro *et al*., 2015), we confirm now that BCO affects fungal cells at several levels, and identify metal scavenging as the main mode of action leading to apoptotic cell death. This highly complex and multitarget mode of action, unlikely to promote fungal resistance, combined with its natural origin and the absence of mammal topical toxicity, or genotoxicity may constitute a stepping–stone into a new era of clinical antifungal agents.

4.2 Material and Methods

4.2.1 Ethics Statement. All assays involving mammals were conducted in two laboratories with GLP compliance status: Covance Laboratories (England), and Eurofins PSL - Product Safety Labs (USA). At Covance, the following studies were performed in accordance with the requirements of the Animals (Scientific Procedures) Act 1986 and all protocols were previously subjected to the site Ethical Review Process (EPR). For the 21-day study, animal testing was performed under the approval of the Research Ethics Committee (UK Health Departments' Research Ethics Service, National Health Service, England), with the project licence number PPL 70/7602-2. At Eurofins PSL, all acute toxicological studies were performed in accordance with the requirements of 40 CFR Part 160: U.S.EPA (FIFRA), 1989, OECD (as revised in 1997) published in ENV/MC/CHEM (98)17, OECD, Paris, 1998; and EC Directive 2004/10/EC, Official Journal of the European Union, L50/44, Feb. 20, 2004. EUROFINS Product Safety Labs (PSL, USA), is AAALAC accredited, GLP compliant and USDA registered. All protocols of the acute toxicological studies were reviewed and unanimously approved by the Internal Animal Care and Use Committee (IACUC) on the fourteen of May 2010: acute dermal toxicity (PSL protocol #P322), primary eye irritation (PSL protocol #324), dermal sensitization (PSL protocol #P328) and primary skin irritation (PSL protocol #P326).

4.2.2 Microorganisms and culture media. *Saccharomyces cerevisiae* BY4743 and *Candida albicans* CBS 562 were grown in Glucose-Yeast extract-Peptone (GYP) medium (0.5% w/v peptone, 0.5% w/v yeast extract, 2% w/v glucose, 1.5% w/v agar), for 24 h, at 30 °C and 34 °C, respectively. For the assays described here, the media used were: Synthetic Complete (SC) broth with 2% (w/v) glucose (FORMEDIUM) for *S. cerevisiae*, and Potato-Dextrose-Broth (PDB, DIFCO) buffered at pH 7.5 and Yeast extract-peptone-Dextrose medium (YPD: 2% w/v peptone, 1% w/v yeast extract and 2% w/v glucose) for *C. albicans.*

4.2.3 Plant material. *Lupinus albus* L. seeds were purchased from Inveja SAS (France) and were germinated and grown in growth chambers with a photoperiod of 16 h light/8 h dark at 18 °C, for periods up to 10 days. The seed coats were removed and the intact cotyledons were dissected from the axes and stored frozen at -80 °C until needed.

4.2.4 Antifungal agents. Blad-containing oligomer (BCO) was extracted and purified from 8-days-old cotyledons as previously described (Monteiro *et al*., 2010) and stored lyophilized at

room temperature. Stock solutions of AMB, FLC and caspofungin (all from SIGMA) were prepared and stored frozen at -20 °C until used.

4.2.5 Global transcriptome profiling. The transcriptome profiling of *C. albicans* was analyzed by RNA-sequencing (RNA-seq). Two cultures of *C. albicans* were grown as described below in the 'Determination of endogenous ROS production' section, one in the presence of BCO, and the other in its absence (control). After 4 h of incubation, cells were harvested and subjected to a treatment with 0.4 mg/mL lyticase in 50 mM Tris-HCl pH 7.5, 1 M sorbitol, 0.8 M KCl, 10 mM $MgSO_4$ and 15 mM β-mercaptoethanol for 1 h at 30 °C with gentle stirring to digest the cell wall. Spheroplasts were pelleted by centrifugation and their RNA extracted according to the instructions of the RNA isolation kit (RNeasy Mini Kit - QIAGEN) and stored at -80 °C.
Poly(A) mRNA was purified from ca. 25 µg total RNA with two rounds of Dynabeads mRNA DIRECT Micro Purification Kit (Invitrogen). cDNA libraries were constructed with the Ion Total RNA-Seq Kit v2 (Life Technologies) and quantified with Agilent DNA 1000 Kit in the Agilent 2100 Bioanalyzer (Agilent Technologies). The fragments of six barcoded libraries were pooled and clonally amplified by emulsion PCR using the Ion PI Template OT2 200 kit v2 and the Ion OneTouch 2 System (Life Technologies), and the positive Ion Sphere Particles enriched with Ion OneTouch ES (Life Technologies). This procedure was performed twice for a total of two groups of clonally amplified library spheres. Finally, the positive ion spheres were loaded into two Ion PI chip v2 and sequenced in the Ion Proton System (Life Technologies) and Ion PI Sequencing 200 Kit V2 at Genoinseq (Biocant, Cantanhede, Portugal). Ion Proton adapter sequences and low-quality bases were trimmed using the Torrent Suite software (Life Technologies). Duplicate reads were removed using PRINSEQ (Schmieder and Edwards, 2011) and rRNA reads were removed using RiboPicker (Schmieder *et al*., 2012). The remaining reads were mapped using TMAP version 4.0.6 (Life Technologies) against the reference transcriptome of *C. albicans* (genome *Candida albicans* SC5314 A22-s06-m01-r05) (Skrzypek *et al*., data retrieved in April 10th, 2016). Read count was performed by eXpress (https://github.com/adarob/eXpress/) for each condition (Roberts and Patcher, 2012). Gene name and description for each reference transcript were extracted from the Candida Genome Database annotation files (Skrzypek *et al*., data retrieved in April 10th, 2016).
Read counts were uploaded to the Degust (available at http://www.vicbioinformatics.com/degust/) and analyzed using Limma/Voom method,

which was incorporated into Degust. Coding sequences with a false discovery rate (FDR) <0.1 and a fold change greater than 1, were considered significant.

4.2.6 *S. cerevisiae* HIP HOP profiling with a panel of gene deletion signature strains. In this study we used *S. cerevisiae* as model organism, rather than *C. albicans*, since this is the only yeast species for which a complete deletion mutant strain collection exists (Giaever and Nislow, 2014). BCO was tested on *S. cerevisiae* gene deletion strains, purchased from EUROSCARF (Supplementary Table S4.1). The 27 selected strains have been previously determined to be characteristic for common modes of action in which a specific target protein is lacking (Hoepfner *et al.*, 2014) and constitute a mini HIP HOP assay (Prescott and Panaretou, 2017). Heterozygous diploids: *nmd3Δ/NMD3, rei1Δ/REI1, lsg1Δ/LSG1, ssl2Δ/SSL2, neo1Δ/NEO1, tim54Δ/TIM54, pik1Δ/PIK1, cmd1Δ/CMD1, tda10Δ/TDA10, mia40Δ/MIA40, tom40Δ/TOM40* and *mrpl19Δ/MRPL19.* Homozygous diploids: *lem3Δ/lem3Δ, fen1Δ/fen1Δ, lro1Δ/lro1Δ, aft1Δ/aft1Δ, ftr1Δ/ftr1Δ, ctr1Δ/ctr1Δ, fet3Δ/fet3Δ, ire1Δ/ire1Δ, hac1Δ/hac1Δ, sur4Δ/sur4Δ, slt2Δ/slt2Δ, aro1Δ/aro1Δ, trp4Δ/trp4Δ, bck1Δ/bck1Δ* and *rim101Δ/rim10 Δ*. The diploids have the following isogenic background: *MATa/α; his3Δ1/his3Δ1; leu2Δ0/leu Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0* with genes deleted with *KANMX4*. Cultures of each strain were grown to late log phase and their optical density determined with a 1cm path length spectrophotometer. The cultures were then adjusted to OD_{600} 0.00015 in SC medium and transferred to a transparent polystyrene 384 well microplate. Each microplate well contained 50 µl yeast culture in liquid SC medium with or without BCO. The microplate with lid was then incubated at 30 °C in a Tecan Infinite M200 plate reader recording OD_{600} readings in 9 separate locations per well every 20 mins between 5 and 27 h. Growth inhibition over 24 h was determined for each strain relative to the corresponding untreated control. The same procedure was carried out for the isogenic control strain BY4743. Each curve is an average of 12 replicate wells.

4.2.7 Effect of metallic ions on BCO bioactivity. To test the effect of metallic ions on BCO antifungal activity, susceptibility tests were performed on *C. albicans* in the presence of increasing concentrations of these elements. The susceptibility tests were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guideline M27-A2 (NCCLS, 2002), with some adjustments, using broth microdilution method, as previously described (Pinheiro *et al*., 2016). Six different

cations were supplied, separately, in the culture medium (PDB): copper ($CuCl_2$ and $CuSO_4.5H_2O$), calcium ($CaCl_2.2H_2O$, $CaSO_4$ and $Ca(NO_3)_2$), magnesium ($MgCl_2.10H_2O$ and $MgSO_4.7H_2O$), iron ($FeCl_2$ and $FeSO_4.7H_2O$), zinc ($ZnCl_2$ and $ZnSO_4.7H_2O$) and manganese ($ZnCl_2$ and $ZnSO_4.7H_2O$). Each cation was tested under different salt forms (described above in round brackets) separately, in order to determine any possible influence of the anion in the results. First, the minimum toxic concentration for each of these cations was determined with concentrations ranging from 4.88 µM to 10 mM. After determining the toxicity lower limit, progressively lower concentrations of each cation were tested with BCO. A culture medium without supplemented cations and with BCO was used as control. All assays were performed in triplicate. The content of each cation in the unsupplemented (original) PDB medium was quantified prior to the addition of the salts. A mixture of three different batches of PDB was homogenized and a single analysis was performed. The mixture was previously acid digested with micro-waves, and the content of the cations was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (ISO 11885, 2007).

4.2.8 Quantification of the plasma membrane ergosterol. A fresh culture of *C. albicans* was inoculated in 50 mL YPD medium for 24 h at 34 °C, without shaking containing a sub-inhibitory concentration of BCO (2.4 µM). Sub-inhibitory concentrations of FLC (208 µM), AMB (0.27 µM) and caspofungin (26 µM) were also tested as references of ergosterol targeted modes of action (positive control - FLC; negative control - caspofungin; multitarget mode of action – AMB). Cells were harvested by centrifugation and washed with distilled water. Total intracellular sterols were extracted and quantified as previously described (Arthington-Skaggs *et al*., 1999). Statistical analysis was performed using GraphPad Prism software version 5.02, La Jolla, California, USA. *P* values were calculated using one-way ANOVA and Bonferroni multiple comparison post- test.

4.2.9 Determination of endogenous ROS production. A fresh culture of *C. albicans* was inoculated ($1x10^5$ CFU/mL) in PDB pH 7.5 with 2.4 µM BCO and incubated at 34°C without shaking. After 4 h incubation, intracellular reactive oxygen species (ROS) levels were measured by a fluorometric assay using the fluorescent dye 2,7-dichloro-dihydro-fluorescein diacetate (DCFH-DA; Sigma) as a ROS indicator, as previously described (Favre *et al*., 2008). PDB pH 7.5 was used as negative control and PDB pH 7.5 with 20 mM H_2O_2 (added 30 min before ROS analysis) as positive control.

4.2.10 Annexin V and PI staining. Annexin V/propidium iodide (PI) binding assays were performed according to the FITC Annexin V/Dead Cell Apoptosis Kit (Molecular Probes), with minor modifications, as previously described (Du *et al*., 2008). Two cultures of *C. albicans* were grown as described in the 'Determination of endogenous ROS production' section, one in the presence of BCO, and the other in its absence (control).

4.2.11 Toxicological studies in mammals. Acute toxicity. 1.1 Dermal (according to the guideline OECD 402, US EPA OPPTS 870.1200) (OECD, 1987; United States Environmental Protection Agency, 1998a). 1.2 Eye irritation (according to the guideline OECD 405, US EPA OPPTS 870.2400) (OECD, 2002b; United States Environmental Protection Agency, 1998b). 1.3 Skin irritation (according to the guideline OECD 404, US EPA OPPTS 870.2500) (OECD, 2002a; United States Environmental Protection Agency, 1996). 1.4 Skin sensitisation (according to the guideline OECD 406, US EPA OPPTS 870.2600) (OECD, 1992; United States Environmental Protection Agency, 2003). **Short-term toxicity**. BCO short-term toxicity was investigated in a 22-day topical dermal toxicity study (guideline OECD 410, 1981) (OECD, 1981). ***In vitro* genotoxicity testing.** 3.1 Bacterial assay for gene mutation – Ames test (according to the guideline OECD 471, 1997) (OECD, 1997a). In a reverse gene mutation assay in bacteria, *Salmonella typhimurium* histidine-requiring strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tryptophan-requiring strain WP2*uvr*A pKM101 were exposed to BCO. Due to the protein nature of the test article, the treat and plate methodology was used in preference to the standard plate-incorporation test, to prevent artifacts due to growth stimulation following the administration of a polypeptide-containing test article. 3.2 Mammalian assay for gene mutation – mouse lymphoma assay (according to the guideline OECD 476, 1997, also compliant with OECD 490, 28 July 2015) (OECD, 1997b; OECD, 2015). 3.3 Mammalian assay for clastogenicity – p53 competent human lymphocyte assay (according to the guideline OECD 487, 2014) (OECD, 2014a). BCO was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from blood samples pooled from two male donors in a single experiment. ***In vivo* studies in somatic cells.** Comet assay (according to the guideline OECD 489, 2014) (OECD, 2014b). This *in vivo* alkaline single cell gel electrophoresis assay, also called alkaline Comet Assay, is a method to measure DNA strand breaks in eukaryotic cells. Based on their size, DNA fragments migrate in the gel away from the head to the tail, and the intensity of the comet tail relative to the total intensity (head plus tail) reflects the amount of DNA breakage.

4.2.12 Fluorescence microscopy. All samples were observed under a fluorescence microscope (Axioscope A1 with phase contrast and epi-fluorescence, Zeiss) equipped with a camera (AxioCam ICm1, Zeiss), using three different filters: Filter Set 49 DAPI (Excitation G 365, Emission BP 420/470); Filter Set 10 FITC/GFP (Excitation BP 450-490, Emission BP 515-565); and Filter Set 15 Rodhamine (Excitation BP 540-552, Emission LP 590).

4.3 Results

4.3.1 BCO effects on *C. albicans* transcriptome and on *S. cerevisiae* mutants. To analyze BCO genome-wide effects and to identify the mechanisms underlying its fungal growth inhibition (Pinheiro *et al*., 2016), RNA-seq was employed to analyze *C. albicans* transcriptome upon exposure to BCO for 4 h. A total of 124 genes were found to be differentially expressed. Of these, 80 were up-regulated and 44 were down-regulated comparatively to untreated yeast cultures. The functional distribution of BCO responsive genes is shown in Figure 4.1.

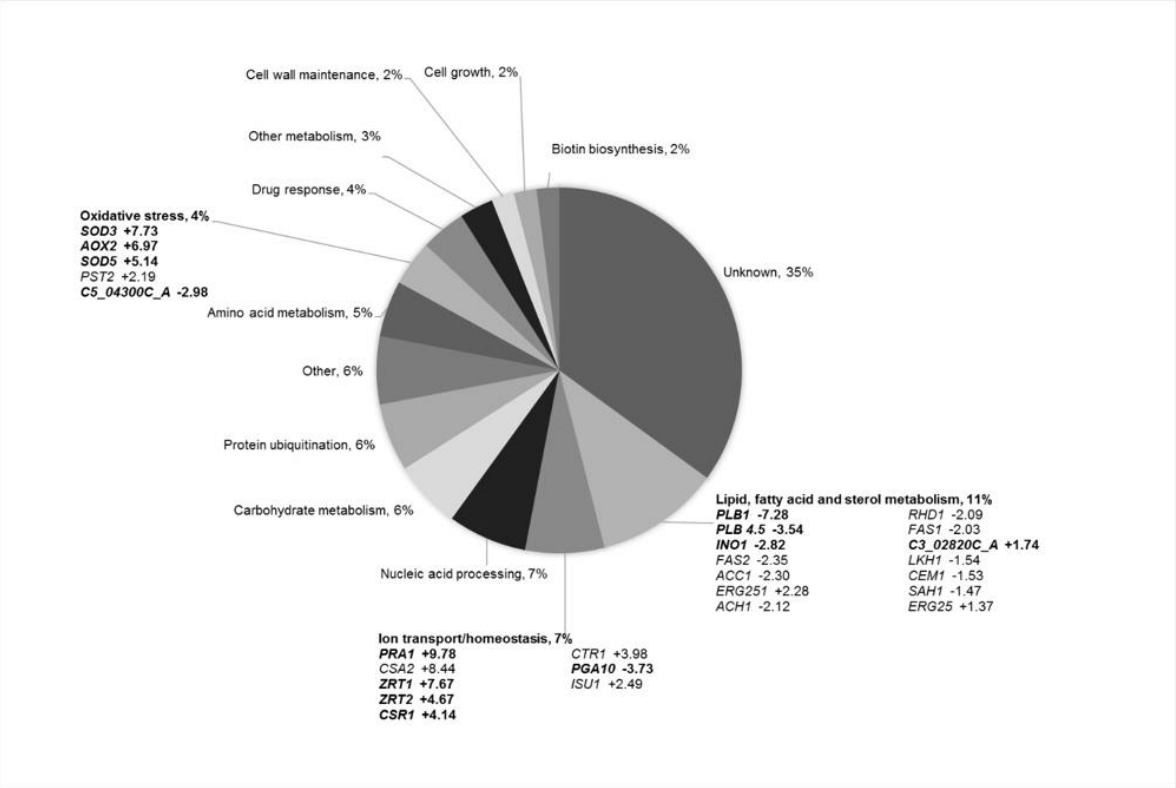


Figure 4.1. Assigning BCO responsive genes from *C. albicans* to functional categories. The average logarithmic (log_2) fold change ratio from three independent experiments is shown. Genes in boldface have a FDR<0.05. All other genes have a FDR<0.1.

To confirm some of these results, a yeast chemical genetics approach was carried out using a short list of *S.cerevisiae* mutant strains based on their known hypersensitive profiles. The selected 27 profiles corresponded to ten common modes of action described for antifungal drugs (Supplementary Table S4.1). Each yeast deletion strain was screened for hypersensitivity to BCO, as well as the parental strain, using a previously determined (Supplementary Figure S4.1) sublethal dose of BCO (0.024 μM). The results presented in Figure 4.2 clearly show that, under these conditions, the only deletion strain displaying a marked hypersensitivity to the

BCO sublethal concentration corresponded to the mutant lacking the transcription factor Aft1, a gene involved on iron utilization and homeostasis (Yamaguchi-lwai *et al*., 1995).

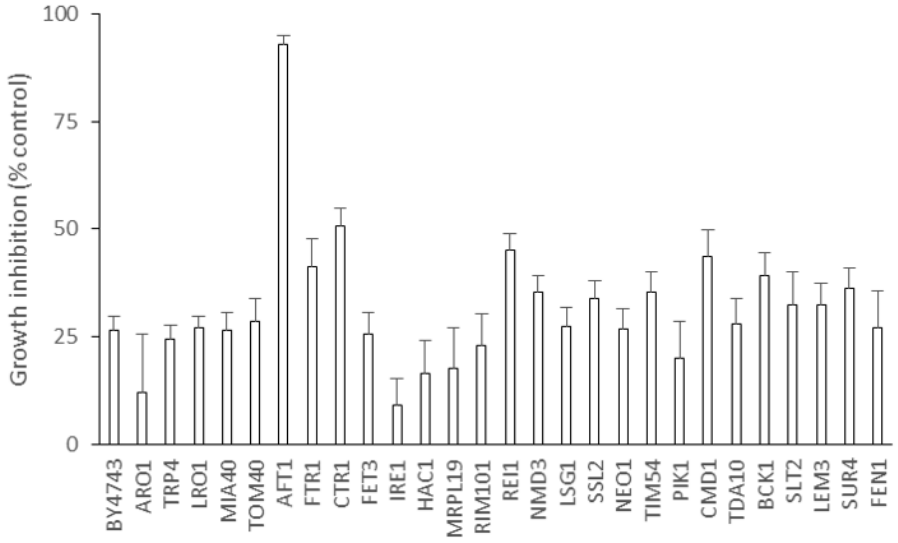


Figure 4.2. BCO induced growth inhibition in a panel of yeast HIP HOP "signature strains" that are characteristic for common modes of action. Signature genes were identified previously through high-throughput genome wide HIP HOP screening of a library of yeast inhibitory small molecules. Growth inhibition of the 27 strains over 24 hours was determined in the presence of a sublethal inhibitory dose of BCO (0.024 μM). Cultures of each strain were diluted to OD_{600} 0.00015 and grown in a 384 well plate with and without BCO. Readings were taken every 20 mins for 27 h to produce 48 separate growth curves. Increase in growth over 24 h was then used to determine growth inhibition of each strain relative to the untreated control of the same genetic background. The same procedure was carried out for the isogenic control strain BY4743. Error bars are SEM, n=12.

4.3.2 Neutralization of BCO activity by divalent cations. Considering the results obtained in the experiments above, namely the hypersensitivity of the yeast mutant strain *aft1Δ/aft1Δ* and the effect on ion transport/homeostasis responsive genes, susceptibility tests to BCO were conducted with *C. albicans* cells in PDB medium supplemented with six divalent cations. The following concentrations of each cation was quantified in the original (unsupplemented) PDB medium: 8 μM Cu^{2+}, 18 μM Ca^{2+}, 59 μM Mg^{2+}, 2 μM Fe^{2+}, 3 μM Zn^{2+} and 0.09 μM Mn^{2+}. These concentrations are negligible compared to the concentrations added and therefore, only the supplemented concentration was considered for the analysis of the results (Table 4.1).

Table 4.1. Effect of different cations on the BCO growth inhibiting activity upon *C. albicans* cells in PDB medium.

Cations	Maximum nontoxic cation concentration (µM)*	Concentration of cation supplemented (µM)	MIC of BCO (µM)	MFC of BCO (µM)**	Maximum cation concentration with no influence in BCO activity (µM)*
-	**-**	-	0.19	0.38	-
Cu^{2+}	1,250	1,250	> 0.76	-	15.62
		625	> 0.76	-	
		125	0.76	> 0.76	
		62.5	0.38	0.76	
		15.62	0.19	0.38	
Ca^{2+}	≥ 10,000	10,000	> 0.76	-	15.62
		1,000	> 0.76	-	
		125	0.76	> 0.76	
		31.25	0.38	0.76	
		15.62	0.19	0.38	
Mg^{2+}	≥ 10,000	10,000	> 0.76	-	125
		1,000	0.38	> 0.76	
		500	0.19	> 0.76	
		250	0.19	> 0.76	
		125	0.19	0.38	
Fe^{2+}	1,250	1,250	> 0.76	-	15.62
		625	> 0.76	-	
		125	> 0.76	-	
		62.5	0.76	0.76	
		15.62	0.19	0.38	
Zn^{2+}	156	156.2	> 0.76	-	3.91
		78.1	0.76	> 0.76	
		15.62	0.38	0.76	
		7.81	0.38	0.76	
		3.905	0.19	0.38	
Mn^{2+}	625	625	> 0.76	-	7.81
		312.5	> 0.76	-	
		62.5	0.76	> 0.76	
		31.25	0.38	0.76	
		15.62	0.38	0.76	
		7.81	0.19	0.38	

* Concentration calculated based only on the added amount of each cation. Each cation was tested independently under two or three different salt formulations (Cu^{2+}: $CuCl_2$ / $CuSO_4.5H_2O$; Ca^{2+}: $CaCl_2.2H_2O$ / $CaSO_4$ / $Ca(NO_3)_2$; Mg^{2+}: $MgCl_2.10H_2O$ / $MgSO_4.7H_2O$; Fe^{2+}: $FeCl_2$ / $FeSO_4.7H_2O$; Zn^{2+}: $ZnCl_2$ / $ZnSO_4.7H_2O$ and Mn^{2+}: $MnCl_2.4H_2O$ / $MnSO_4$). There was no variation among triplicates of the MIC and MFC tests.
** (-) MFC was not determined because there was no well without growth.

Considering the inherent toxicity of each cation to the cells (Table 4.1), sub-toxic concentrations were tested to assess their individual effect on the activity of BCO against *C. albicans*. As shown in Table 4.1, all cations induced a marked increase in both MIC and MFC values, thus expressing a clear decrease in the antifungal activity of BCO. Ultimately, albeit at different concentrations, all cations were able to neutralize the activity of BCO. The cation potency to neutralize BCO was lower for magnesium, followed by copper, calcium and iron. Manganese and specially zinc were the cations with the strongest influence on BCO. No differences were found for distinct salts of the same cation nor between replicates.

4.3.3 BCO interferes with the yeast cell membrane ergosterol content. The set of genes in the category of lipid, fatty acid and sterol metabolism was among the most responsive in the RNA-seq experiment (Fig. 4.1) and, therefore, the effect of BCO on the ergosterol content of *C. albicans* plasma membrane was assessed. As a positive control, total ergosterol content was also determined after exposing the cells to FLC. AMB was used as a reference control for a cell membrane disruption mode of action without direct interference in the biosynthesis of ergosterol. Caspofungin was used as negative control.

The results (Fig. 4.3) show that *C. albicans* suffered a 91% reduction in its ergosterol contect when exposed to FLC, as compared to the control. This reduction was significantly lower when the culture was exposed to AMB (71%) or BCO (69%) whereas caspofungin did not interfere with the ergosterol content at all. No statistical differences were observed in the reduction of the ergosterol content between AMB and BCO.

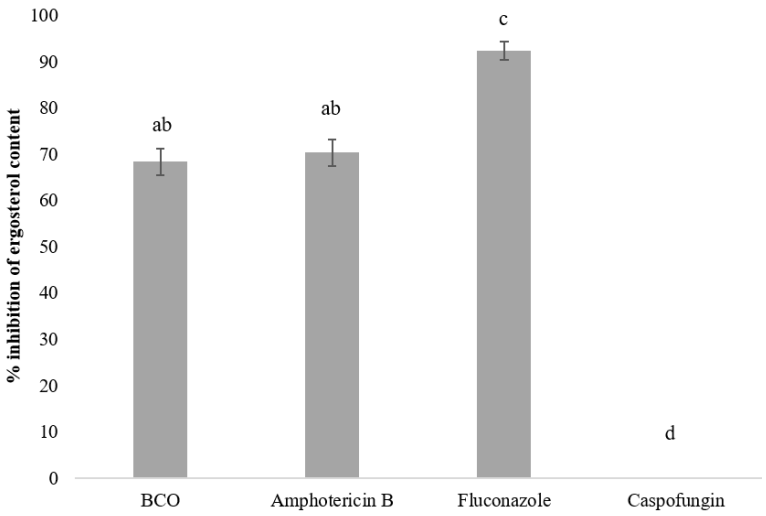


Figure 4.3. Effect of sub-inhibitory concentrations of BCO, AMB, FCL and caspofungin on the inhibition of ergosterol synthesis in *C. albicans*. The results represent the mean ± standard deviation of three independent experiments. Statistical analysis was performed as described in the Material and methods section. Bars with a letter in common are not significantly different (P>0.05).

4.3.4 Induction of C. albicans apoptosis by BCO. The third interesting set of responsive genes obtained with the RNA-seq study was the oxidative-stress associated genes (Fig. 4.1). This gene family has long been associated to cell apoptosis and previous studies have already shown the induction of cell death by BCO (Pinheiro *et al*., 2016). To investigate whether BCO lethal effect occurs via induction of apoptosis, and/or necrosis, annexin V and propidum iodide (PI) staining was performed (Fig. 4.4). An early marker of apoptosis is the exposure of phosphatidylserine (PS) on the cell surface, since it is normally located in the luminal layer of the cytoplasmic membrane (Martin *et al*., 1995; Madeo *et al*., 1997). Annexin V displays a high affinity for PS and its previous labeling with Fluorescein isothiocyanate (FITC) allows the visual identification of apoptotic cells under a fluorescence microscope. Staining with PI allows detection of damaged membranes. Therefore, differential staining patterns discriminate among live cells (annexin V-/PI-), early apoptosis (annexin V+/PI-), necrosis (annexin V-/PI+), and late apoptosis/necrosis (annexin V+/PI+) (Hao *et al*., 2013).

The majority of *C. albicans* cells exposed for 4 h to 2.4 μM BCO were only green in color, which is indicative of early apoptosis (Fig. 4.4A). Some cells were stained with both dyes, meaning that, at this point, a minority of cells was undergoing late apoptosis (Figs. 4.4A and 4.4B). Cells

grown in the absence of BCO were not stained by annexin V or PI (data not shown).

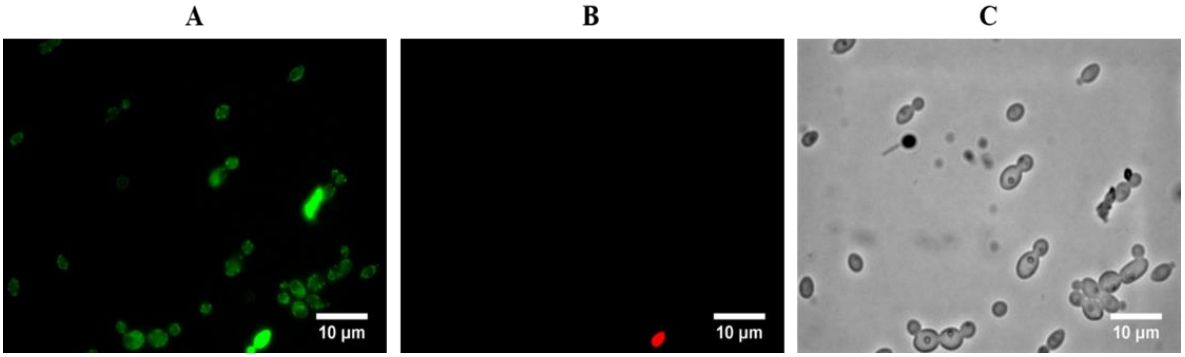


Figure 4.4. Induction of *C. albicans* apoptosis by BCO. Annexin V and propidium iodide staining of *C. albicans* cells exposed to 2.4 µM BCO for 4 h. Cells labeled with annexin V (**A**), propidium iodide (**B**), or simply observed by bright field microscopy (**C**). Bar corresponds to 10 µm.

4.3.5 ROS are produced during BCO induced apoptosis. Production of reactive oxidative species (ROS) by *C. albicans* exposed for 4 h to 2.4 µM BCO was analyzed with DCFH-DA, a nonfluorescent ROS indicator which diffuses across the cell membrane, undergoes oxidation and emits green fluorescence (Fig. 4.5). Appropriate controls were cells grown in the absence of BCO (negative control) and cells exposed to 20 mM H_2O_2 for 30 min (positive control). After 4 h of incubation with BCO, ROS accumulation was already detectable in some cells (Fig. 4.5A). The negative control did not present DCFH-DA staining, whereas in the positive control 100% cells were stained (data not shown).

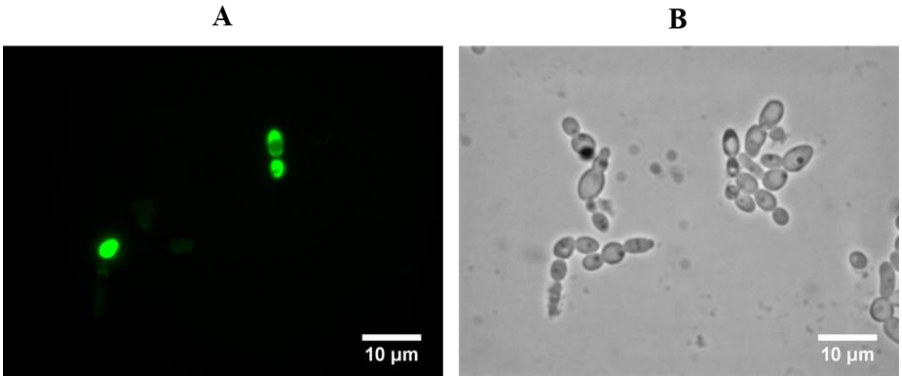


Figure 4.5. Detection of endogenous ROS production. DCFH-CA staining of C. albicans cells exposed for 4 h to 2.4 µM BCO. Cells labeled with DCFH-DA (**A**) or simply observed by bright field microscopy (**B**). Bar corresponds to 10 µm.

4.3.6 Absence of topical toxicity (acute and short term) and genotoxicity upon exposure of mammals to BCO (*in vitro* and *in vivo* assays)

Acute toxicity. No gross signs of clinical toxicity, adverse pharmacologic effects or abnormal behavior were observed following dermal (10 rats), eye irritation (3 New Zealand White rabbits), skin irritation (3 New Zealand White rabbits) and skin sensitization (20 Guinea pigs). There were also no signs of toxicity observed at necropsy in all tests. A summary of the overall results for BCO acute toxicity is shown in Table 4.2.

Table 4.2. Summary of acute toxicity of BCO.

Type of study	Species	Result
Dermal	Rat	LD_{50}: 400 mg BCO/kg
Skin irritation	Rabbit	Slightly irritating
Eye irritation	Rabbit	Mildly irritating
Skin sensitisation	Guinea pig	Non-sensitising

LD_{50} – median lethal dose

BCO gave rise to non-toxic classification resulting from rat dermal (LD_{50} 400 mg BCO/kg). Although the skin and eye irritation studies showed a slightly or mildly irritating effect, these results were insufficient to warrant classification according to Annex I of Regulation (EC) No. 1272/2008 (of the European Parliament and of the Council of 16 December 2008 on the classification, labelling and packaging of substances and mixtures). Furthermore, BCO was not a skin sensitizer in guinea pig, using the Buehler method.

Short-term toxicity. In the 22-day dermal test, no clinical observations of toxicological importance, no deaths and no clinical signs of toxicity at necropsy were noted in all groups of rats tested (Crl:WI (Han) rats, $5.sex^{-1}.dose^{-1}$).

Genotoxicity testing. These results are summarized in Table 4.3.

Table 4.3. Summary of genotoxicity testing of BCO.

Type of study	Test system	Dose range tested[a]	Result[a]
In vitro	Bacterial (5 strains, Ames test, treat and plate methodology)	+/-S9: 3.2 to 1000 µg/plate	±S9: negative
In vitro	Mammalian (L5178Y *TK*$^{+/-}$) gene mutation	Experience 1: 3h –S9: 100 to 400[b] µg/mL 3h +S9: 30 to 180[c] µg/mL Experience 2: 3h –S9: 100 to 500[c]µg/mL 3h +S9: 60 to 240[c]µg/mL	-S9: negative +S9: positive
In vitro	Mammalian (cultured human lymphocytes) micronucleus	3 (+21) h +/-S9: 0 to 400 µg/mL 24 (+24) h –S9: 20 to 320[c] µg/mL	±S9: negative
In vivo	Rat stomach comet	0, 100, 200, 400 mg/kg bw/day	Negative

[a] S9 from rat liver
[b] Higher concentration tested without precipitate at the end of treatment
[c] Concentration limited by toxicity

***In vitro* genotoxicity testing.** Bacterial assay for gene mutation: No evidence for BCO induced mutagenic activity was detected in the system tested (three *Salmonella typhimurium* histidine-requiring strains, and one *Escherichia coli* tryptophan-requiring strain) either in the presence nor in the absence of a rat liver metabolic activation system (S9). Mammalian assay for gene mutation: In the absence of the activation system S9, BCO did not induce gene mutation. In the presence of S9, there was an increase in mutant frequency (MF) that exceeded the sum of the Global Evaluation Factor (GEF) + negative control for the highest concentrations tested. In those cases, increases in both small (predominantly) and large colony mutant frequencies were observed. Under the conditions of this study the data constituted a positive result in the presence of S9 and indicated the possibility that the increase in MF observed is potentially being driven by a clastogenic type of mechanism. However, the clastogenicity assay performed in mammalian cells (with cultured human lymphocytes) was negative.

***In vivo* genotoxicity testing.** Comet assay. There was no dose-related increase in % hedgehogs in stomach following treatment with BCO, thus demonstrating that treatment with BCO did not cause excessive DNA damage (which can interfere with Comet analysis) following oral gavage

administration, nor did mechanical/enzyme-induced damage result during sample preparation. BCO did not originate an increase in the incidence of DNA damage, as measured by % tail intensity in the stomach of male rats (20/dose), following oral dosing up to a level of 400 $mg.kg^{-1}$ $bw.day^{-1}$.

4.4 Discussion

The limited repertoire of antifungal drugs to treat human fungal infections, further restrained by increasing events of host toxicity and drug resistance, has created an urgent and desperate need for new drugs with novel mechanisms, for both agricultural and clinical applications. The most up-to-date research for antifungal drug discovery is focused in alternative treatments with drugs aimed at novel fungal targets within the cellular circuitry crucial for stress response (Lamoth *et al*., 2014), drug resistance (Holmes *et al*., 2016) and virulence (Vila *et al*., 2017), although the classical research for novel targets aimed at cell survival still prevails (Balouiri *et al*., 2016). Here we describe a novel mode of action of a new, natural and multi-target antifungal compound (BCO) and assess its toxicity towards mammalian cells.

In a genome-wide analysis of the transcriptome from *C. albicans* exposed to BCO, the most representative functional class of responsive genes included lipid, fatty acid and sterol metabolism: three genes involved in sterol biosynthesis (*C3_02820C_A*, *ERG25, ERG251*) were up-regulated, suggesting a response to ergosterol depletion (Liu *et al*., 2005), whereas genes belonging to the lipid (*PLB1, INO1, PLB4.5, RHD1, LKH1* and *SAH1*) and fatty acid metabolisms (*ACH1, FAS2, CEM1, ACC1* and *FAS1*) were all down-regulated. Downregulation of these genes has already been described in *C. albicans* after AMB exposure (Liu *et al*., 2005), and suggests a disruptive effect of BCO at the cell membrane level (Spampinato and Leonardi, 2013). Ion transport/homeostasis seems to play a key role on BCO bioactivity upon *C. albicans* cells, as the expression of genes belonging to this class were also largely affected, particularly those related to zinc homeostasis: upregulation of *CSR1*, *ZRT1*, *ZRT2* and *PRA1* (Citiulo *et al*., 2012; Böttcher *et al*., 2015). The over-expression of these genes is induced by mild zinc deficiency (Wu *et al*., 2008) originated by its putative sequestration. Zinc is, after magnesium, the second most widespread metal present in enzymes belonging to all six major functional classes (Andreini *et al*., 2008), and zinc ions are essential for a wide variety of biochemical processes. Control of zinc homeostasis is especially relevant for pathogens because the amount of labile zinc in host tissues is very low. Accordingly, mammals have developed the capacity to inhibit microbial growth in their tissues by zinc starvation, as part of a broader defense mechanism termed "nutritional immunity" (Hood and Skaar, 2012). Besides zinc, both copper and iron are also required to organisms, primarily through their role as cofactors in essential metabolic functions (Marvin *et al*., 2003; Lan *et al*., 2004). They were both also affected by BCO: *CSA2*, a gene involved in a heme-iron uptake system in *C. albicans* (Weissman and Kornitzer, 2004) was

upregulated, which is in accordance with a low-iron environment, and *CTR1*, a copper ion transporter, was also upregulated, probably due to its essential role in iron uptake (Lan *et al*., 2004; Lee *et al*., 2005). BCO also seems to impose oxidative stress inside yeast cells, as upregulation was also observed on four oxidative stress-related genes: *SOD3* and *SOD5*, *AOX2* and *PST2* (Liu *et al*., 2005; Frohner *et al*., 2009; Li *et al*., 2015). This was corroborated by downregulation of *C5-04300C_A* gene, a homologue of *S. cerevisiae DUG1* gene, which encodes a glutamine amidotransferase involved in glutathione catabolism (Toledano *et al*., 2013), given that glutathione acts as an important line of defense against reactive oxygen species in bio-reductive reactions (Mendoza-Cózatil *et al*., 2005). Genes involved in biotin biosynthesis, namely *BIO2*, *BIO3* and *VHT1* (Chattopadhyay *et al*., 2009) were also downregulated upon exposure to BCO. This may derive from downregulation of genes involved in lipid and fatty acid metabolisms, because phospholipid precursors play an essential role in biotin biosynthesis (Santiago and Mamoun, 2003). In addition, biotin synthesis is also repressed under low iron conditions (Shakoury-Elizeh *et al*., 2004). Either way, this repression in biotin synthesis is most likely a secondary effect of the exposure to BCO rather than one of its primary targets. In the same segment are the genes related to nucleic acid processing, carbohydrate metabolism, protein ubiquitylation, amino acid metabolism, drug response and cell growth.

The effect of metal ions on BCO activity was further elucidated in *S. cerevisiae*, using HIP HOP profiling with a panel of gene deletion signature strains. From all the deletion mutant strains tested, only the homozygous deletion strain *aft1Δ/aft1Δ* displayed a marked sensitivity to BCO. *AFT1* is a transcription factor involved in iron utilization and homeostasis (Yamaguchi-Iwai *et al*., 1995). These results rule out the hypothesis of a direct effect of metals on the activity or structure of BCO, and suggest that interference with iron homeostasis and/or other iron metabolic reactions are involved in BCO mode of action. Previous results have already demonstrated the metal-binding activity of BCO *in vitro* (Table S4.2). It was demonstrated that in the presence of metallic ions the melting temperature of BCO shifts in several degrees allowing to conclude that they are, potentially, strong binding ligands for BCO. Furthermore, a broader BCO cation scavenging activity was observed in susceptibility tests against *C. albicans*, with a complete neutralization of its antifungal activity by several divalent cations (zinc, manganese, iron, copper and calcium).

The alterations in gene expression related to cell membrane and ergosterol, as well as the previously described destabilizing effect of BCO on yeast cell membranes (Pinheiro *et al*., 2016), could indicate an interference of BCO with ergosterol synthesis. This isoprenoid is the main sterol found in fungal and protozoa cell membranes, and is also the target of many well-known drugs, including azoles. However, our results seem to indicate that BCO destabilizes the cell membrane, but not in an ergosterol metabolic pathway-dependent way. Similarly to other antifungals (Munoz *et al*., 2006; Van Der Weerden *et al*., 2008), this seems to be a secondary target of BCO, clearly undermining cell stability, but without an evident effect on cell growth and/or viability *per se*.

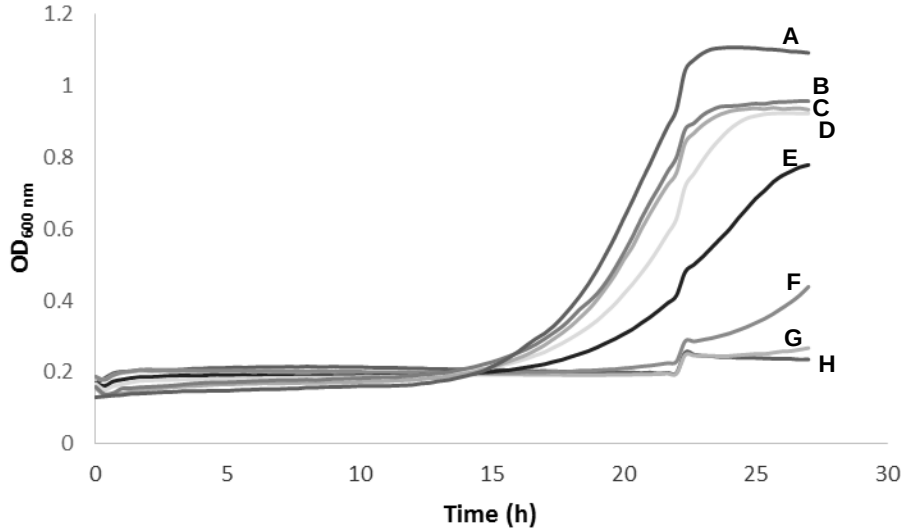
The externalization of PS here demonstrated, combined with the detection of endogenous ROS production and the upregulation of oxidative stress response genes upon exposure of *C. albicans* to BCO, are consistent with an apoptotic cell death. The same mechanism has already been described for AMB (Phillips *et al*., 2003) and for other antifungals (Wu *et al*., 2010; Aerts *et al*., 2011; Emrick *et al*., 2013).

BCO was also thoroughly evaluated in a range of genotoxicity assays performed both *in vitro* and *in vivo*. Concerns raised over the increase in mutant frequency in the mouse lymphoma assay were not replicated *in vitro* in either the Ames study (from a gene mutation mode of action pathway) or in the p53 competent human lymphocyte assay (from a structural chromosomal mode of action pathway). Furthermore, the *in vivo* rat stomach comet was concluded to be devoid of any DNA damage. Overall, it may be concluded from the evidence presented here that BCO is devoid of any genotoxic potential. No carcinogenicity studies have been conducted because there is no evidence in the available literature to suggest that proteins similar to BCO, comprising polypeptide segments of β-conglutin are associated to an increased incidence of cancer. On the contrary, many legume seed proteins have been claimed to exhibit anticancer potential (Quiroga *et al*., 2015). Therefore, BCO is unlikely to be considered a carcinogen.

In summary, we demonstrate here that BCO has a disruptive effect on the cell membrane, allowing its entry into the cell, but which is not ergosterol-dependent. Similarly to its known interference with the cell wall (Pinheiro *et al*., 2016), this does not seem to be its primary antifungal mode of action. The available evidence suggests that BCO primary mode of action is a metal scavenging activity, with a particular effect on zinc and iron, shattering cell homeostasis.

This activity might be exerted both extra- and intracellularly. An oxidative stress is then generated inside the cells, culminating in an apoptotic cell death. BCO showed no evidence of topical toxicity towards mammalian cells after acute or short-term expositions. The absence of topical toxicity, genotoxicity and carcinogenicity of BCO in mammals is of outmost importance to enable its potential application to treat topical fungal infections. Moreover, the metal homeostasis at the host-pathogen interface, which is BCO's primary mode of action, is now recognized as a preferential target for the development of new antifungal drugs, because metal chelators seem to be beyond drug resistance (Polvi *et al*., 2016). Further studies are now required to fully understand its interaction with fungal cells (namely, its mode of cell entrance), and with the human body (toxicity and stability after intravenous administration) in order to determine its potential for the treatment of systemic fungal infections. Nevertheless, BCO is already an undoubtedly powerful, novel and very wide spectrum tool exhibiting multitarget modes of action, and with an apparently very low risk for fungal resistance.

4.5 Supplementary Material



Supplementary Figure S4.1. Growth curves of S. cerevisiae BY4743 parental strain in SC medium supplemented with 2% (w/v) glucose. Each growth curve is the average of 12 replicate wells. The $OD_{600\ nm}$ of each individual well is an average of nine readings made at different locations of the well. The top line (**A**) refers to the untreated culture (absence of BCO) and the other lines represent the growing culture in the presence of increasing BCO concentrations: 0.012 µM, 0.024 µM, 0.047 µM, 0.095 µM, 0.19 µM, 0.38 µM and 0.76 µM (**B** to **H**, respectively).

Supplementary Table S4.1. *S. cerevisiae* deletion strains obtained from Euroscarf. The strains marked HIP (haploinsufficiency profiling) are deleted for one copy of the gene in question. Those marked HOP (homozygous profiling) are deleted for both copies of the gene.

Gene	Systematic Name	Accession Number	HIP or HOP	Likely mode of action
NMD3	YHR170W	Y26418	HIP	DNA intercalation
REI1	YBR267W	Y23407	HIP	
LSG1	YGL099W	Y24466	HIP	
SSL2	YIL143C	Y22302	HIP	
NEO1	YIL048W	Y21441	HIP	Membrane perturbation
TIM54	YJL054W	Y21369	HIP	
PIK1	YNL267W	Y26958	HIP	
CMD1	YBR109C	Y23248	HIP	
TDA10	YGR205W	Y24835	HIP	
BCK1	YJL095W	Y31328	HOP	
SLT2	YHR030C	Y30993	HOP	
LEM3	YNL323W	Y31121	HOP	
SUR4	YLR372W	Y35281	HOP	Membrane perturbation/lipids
FEN1	YCR034W	Y35763	HOP	
ARO1	YDR127W	Y34061	HOP	Plasma membrane stress, internalization of amino acid permeases
TRP4	YDR354W	Y34191	HOP	
LRO1	YNR008W	Y35383	HOP	Fatty acid disruption
MIA40	YKL195W	Y27033	HIP	Mitochondrial membrane perturbation
TOM40	YMR203W	Y20789	HIP	
AFT1	YGL071W	Y34438	HOP	Cation chelation
FTR1	YER145C	Y36142	HOP	
CTR1	YPR124W	Y35539	HOP	
FET3	YMR058W	Y36192	HOP	
IRE1	YHR079C	Y31907	HOP	ER stress
HAC1	YFL031W	Y35650	HOP	
MRPL19	YNL185C	Y22027	HIP	Uncoupling
RIM101	YHL027W	Y30936	HOP	pH stress

Supplementary Table S 4.2. Melting temperatures of BCO in the presence of 10 mM of different metallic ions**.**

Additives	**Melting temperature (°C)**
Control (H_2O)	68.5; 73.5
$MgCl_2$	83
$CaCl_2$	85
$MnCl_2$	86
$ZnCl_2$	62.5; 82.5
$ZnSO_4$	64.5; 82.5
$NiCl_2$	86

The results in Supplementary Table S4.2 were obtained by Thermal shift (Thermofluor) assay using BCO at 0.25 µg/µL, as described in (Boivin *et al*., 2013). When in water, BCO presents two melting temperatures, which corresponds to two different unfolded stages. This is probably due its nature as an oligomer and may indicate multiple denaturation components. The addition of the divalent metallic ions had a stabilizing effect on the BCO, considering the significant increased on its melting temperature. This means that the BCO-metallic ions binding stabilized the oligomer by minimizing its aggregation and increasing the temperature of denaturation.

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Chapter 5

High-throughput expression and preliminary structural characterization of Blad polypeptide

Pinheiro AM, Ferreira RB and Monteiro SA (201x) High-throughput expression and preliminary structural characterization of Blad polypeptide. (Manuscript in preparation).

Chapter 5 - High-throughput expression and preliminary strutural characterization of Blad polypeptide

Abstract

Blad polypeptide occurs naturally as a major subunit of an edible, 210 kDa oligomer (Blad-containing oligomer - BCO), and previous studies have shown that BCO has excellent antifungal activity and lacks toxicity, genotoxicity and carcinogenicity in mammals. Among the oligomer subunits, Blad is the only one exhibiting lectin activity. This observation prompted us to assume that Blad is the mainly responsible for the antifungal activity of BCO, leading to an attempt of Blad production in recombinant form. The heterologous expression of Blad has been challenging due to its unusual solubility characteristics, which has also been responsible for delaying its structural characterization. In an attempt to circumvent Blad poor solubility and using a high throughput screening approach, four different fusion proteins and six-Blad based clones where tested. Finally, the His_6-MBP-Blad was selected as the most promising construct for proper expression and purification. Oligomers of His_6-MBP-Blad were first analyzed by Dinamic light scattering, negative-staining electron microscopy and Small angle X-ray scattering, which revealed that the fusion protein was structurally homogeneous and seemed to have an estimated molecular weight of approximately 1.15 MDa, suggesting that each particle was constituted by 18 monomers of the fusion protein. Crystals of the recombinant fusion protein were tested for X-ray diffraction analysis and the structure of the fusion protein was solved with a maximum resolution of 3.5 Å. After failing to apply the molecular replacement technique, using the tridimensional structure of MBP and a theoretical model of Blad, a tridimensional model of a heptameric protein with a molecular weight similar to that of His_6-MBP-Blad was used. Surprisingly, the refinement and optimization of the model revealed the presence of two molecules of the bacterial chaperonine GroEL in each asymmetric unit of the crystal. Host chaperone protein is a common contaminant that is often co-purified with tagged proteins, however, considering that the molecular mass of GroEL is practically the same as that of His_6-MBP-Blad (approximately 60 kDa), it was impossible to detect the contamination on any SDS-PAGE. After several attempts to remove the contamination it persisted and so, in the future, a different approach must be followed to allow the production of recombinant Blad, in soluble form, and without any host protein contamination.

Keywords: Blad, *Escherichia coli*, heterologous expression, tridimensional structure

5.1 Introduction

Knowledge of the three dimensional structure of a protein is a valuable asset for better understanding its mode of action (Anfinsen, 1973; Nanni *et al*., 2014). X-ray diffraction from high quality crystals remains the most reliable approach to obtain detailed structural information that provides powerful insight into the molecular mechanisms underlying the function of bio-macromolecules and the way they interact to form complex supramolecular assemblies (Krauss *et al*., 2013; Bai *et al*., 2014). Before crystallization can be attempted, the target protein must be purified from the source or, more often, produced in a recombinant form. However, recombinant production and purification are serious bottlenecks that must be tackled to secure the hundreds-of-milligram quantities necessary, in a stable form, for a successful structural biology project (Bill *et al*., 2011).

Fusion partners have become powerful tools to simplify the proper expression and purification of recombinant proteins (Sørensen and Mortensen, 2005a; Stevens, 2000). Apart from facilitating the purification process, like polyhistidine (poly-His), a tag that allows a one-step affinity purification (Rosano and Ceccarelli, 2014), affinity tags can also enhance protein solubility and stability (Pina *et al*., 2014; Sørensen and Mortensen, 2005b) with a wide range of fusion partners being developed. Among the most popular solubility-enhancer partners, are the maltose-binding protein (MBP) (Kapust and Waugh, 1999), N-utilization substance protein A (NusA) (Davis *et al*., 1999) and thioredoxin (Trx) (LaVallie *et al*., 1993).

Blad polypeptide occurs naturally as a major subunit of an edible, 210 kDa oligomer (Blad-containing oligomer - BCO), which accumulates to high levels, exclusively in the cotyledons of *Lupinus* seedlings between the 4th and 14th day after the onset of germination. Previous studies have shown that BCO has excellent antifungal activity and lacks toxicity, genotoxicity and carcinogenicity in mammals (Monteiro *et al*., 2015; Pinheiro *et al*., 2016). Evidence that BCO is a multitarget drug might be a step stone into a new era of clinical antifungal agents (Pinheiro *et al*., 2017). Among the oligomer subunit, Blad is the only one exhibiting lectin activity, as evidenced by its ability to bind antibodies and other glycoproteins (Ramos *et al*., 1997). Blad lectin activity is extremely resistant to inactivation, showing a very high stability against denaturation, treatment with organic solvents and detergents, and exposure to high concentrations of strong acids (Monteiro *et al*., 2015). These results and other experiments prompted us to assume that Blad is responsible for BCO antifungal activity.

The possible application of Blad in clinical fields, especially for treatment of systemic fungal infections, will require its use as a purified active ingredient, free from the other BCO subunits. The great similarity among BCO subunits and the great Blad water insolubility prevented us from isolating it from the other subunits using chromatographic techniques. Therefore, Blad production in a recombinant form by heterologous expression is an approach that had to be considered. Heterologous expression of natural products enables the (over)production of structurally complex substances through transfer of the biosynthetic genes from the original producer to more amenable heterologous hosts (Wenzel and Mu, 2005). Despite advances on the functional aspects of Blad, the challenges on its heterologous expression and purification have been delaying its structural characterization. The primary sequence of a given protein is the most determinant factor of protein solubility (Idicula-thomas *et al*., 2006), specifically in what concerns number and position of the hydrophobic residues in the protein structure (Luan *et al*., 2004). Blad sequence is mainly composed of hydrophilic amino acids and a quick analysis of the distribution of its polar and apolar residues along the protein sequence reveals that Blad presents a highly soluble chart profile, possessing only two distinct hydrophobic regions in the middle of its primary sequence. However, Blad is highly insoluble upon heterologous expression, exhibiting a behavior similar to those of hydrophobic molecules, meaning that its primary structure is not the key reason for its high level of insolubility upon heterologous expression. Several attempts have been made in order to overcome this problem, always without success.

Here we report a high throughput screening approach to rapidly identify the optimal conditions for expression and purification of recombinant Blad. Also, an attempt was made to provide a deep structural characterization of the polypeptide Blad, gathering data from electronic microscopy, dynamic light scattering, SAXS and X-ray crystallography.

5.2 Materials and Methods

5.2.1 Strains and media. DH5α™ competent *Escherichia coli* cells were used for all routine cloning experiments. For recombinant protein overexpression, three different *E. coli* strains were used, BL21 Star™ (DE3), C41 (DE3) and Rosetta™ (DE3) pLysS, all cultivated in LB (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) and TB (12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4 and 4 mL/L glycerol) media.

5.2.2 Plasmids and oligonucleotides. Plasmids and oligonucleotides used in this study are listed in Tables S1 and S2. General cloning procedures were performed as described by Scholz and colleagues (Scholz *et al*., 2013). The correct nucleotide sequence of the inserts in all the constructed plasmids was checked by DNA sequencing.

5.2.3 Design of the new Blad-based clones. All new Blad-based clones were obtained by site-directed mutagenesis according to the QuikChange II Site-Directed Mutagenesis kit (Agilent Technlogies) instructions.

5.2.4 Expression of constructs. Cells harboring plasmids encoding the different constructs were grown overnight at 37 °C with shaking, in liquid media (LB or TB) supplemented with the respective antibiotic: ampicillin for BL21 Star™ (DE3), kanamycin for C41 (DE3) and chloramphenicol for Rosetta™ (DE3) pLysS. For the initial expression screening, 20 µL of the preculture were inoculated in 2 mL of fresh media on a 24-well plate. For production upscaling, 10 mL of the preculture were used to inoculate 1 L of fresh media. Inoculates were grown at 37 °C until $OD_{600\ nm}$ reached 0.4 and then induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were pelleted after 6 h by centrifugation at 4000 *g,* 4 °C, for 20 min.

5.2.5 Fusion protein purification. Cell pellets were ressuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.25 mg/mL lysozyme), submitted to a freeze/thaw cycle and then incubated with DNase (2.5 µg/mL) and $MgCl_2$ (5 mM), thus originating the total fraction. The soluble and insoluble fractions were separated by centrifugation (3500 *g*, 10 min, 4 °C).

The soluble fraction was applied onto a MBPTrap HP column (GE Healthcare), previously equilibrated with 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl and 10 % (v/v) glycerol,

and eluted with 10 mM maltose in binding buffer. The eluted fraction from the MBPTrap HP column was dialyzed against 20 mM Tris-HCl pH 7.5, 150 mM NaCl and injected in a Superose 6 10/300 GL column (GE Healthcare) previously equilibrated in the same buffer.

5.2.6 MBP-tag removal using 3C protease. The oligomers of His_6-MBP-Blad were incubated with the 3C protease for 16 h at 4 °C. The samples were centrifuged and both the soluble (supernatant) and the insoluble (pellet) fraction were analyzed by SDS-PAGE.

5.2.7 Release of GroEL from the fusion protein. The oligomers of His_6-MBP-Blad were incubated with 5 mM MgATP for 30 min and with 10 mM MgATP for 4 h and overnight, all at room temperature. The mixture was then applied onto a chelating Sepharose column equilibrated with 20 mM Tris-HCl pH 8.0 and 0.1 M NaCl and left for 20 min at room temperature with gentle stirring. The column was washed with PBS and the elution of the fusion protein was performed with 20 mM Tris HCl pH 8.0, 0.1 M NaCl and 0.5 M imidazole.

5.2.8 Preliminary structural characterization.

Dynamic light scattering (DLS). Molecular size measurements were carried out at 25 °C in a Zeta sizer Nano Zs DLS system (Malvern Instruments). The sample (~1 mg/mL) was centrifuged and filtered before analysis. Data obtained from three independent measurements were analyzed using the software provided by the manufacturer. The particle size can be determined by measuring the random changes in the intensity of light scattered from particles undergoing random Brownian motion. The correlation curve was fitted to a single exponential form and the RH calculated from the diffusion coefficient (D) using the Stokes-Einstein equation ($RH = kT/6\pi\eta D$, where k is the Boltzmann constant, T is the temperature and η is the medium viscosity) (Berne and Pecora, 2000).

Transmission electron microscopy (TEM). TEM visualization of purified BLAD oligomers was performed using a TEM JEM-1400 (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. Protein samples were diluted in water to 0.08 mg/mL, adsorbed to formvar/carbon-coated 300 mesh nickel grids (FCF300-NI, Electron Microscopy Sciences), negatively stained with 2% (w/v) uranyl acetate, dried and observed at a magnification of 80,000-100,000×.

Small-angle X-ray scattering (SAXS). SAXS data were collected at the SWING beamline (Soleil, France) using a PCCD170170 Aviex detector with radiation of wavelength 1.0332 Å. Protein

samples for SAXS analysis were purified and equilibrated in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol. Data were collected at a sample-to-detector distance of 1.98 m over a scattering-vector range from 0.01 to 0.55 $Å^{-1}$ [$s = (4 \pi \sin\theta)/\lambda$, where 2θ is the scattering angle]. SAXS data for protein samples at 1 and 2 mg/mL and buffer were measured using a batch-mode, in which the scattering patterns of the buffer solutions were recorded before and after of the measurements of each protein sample. No radiation damage was observed by comparison to 10 successive 1 s exposures. Data were processed and analyzed using the ATSAS package (Petoukhov *et al*., 2012).

5.2.9 Crystallization, data collection and processing. Initial crystallization conditions in sitting-drop geometry were identified at 20 °C from drops composed of identical volumes (1 µL) of protein (6 mg/mL in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol) and precipitant solution, equilibrated against a 300 µL reservoir. For initial screening, commercial sparse-matrix crystallization screens were used (e.g. SG1 Shotgun Screen (MD1-88) and Midas (M1-59) from Molecular Dimensions). The protein crystallized in several conditions containing PEG (20-25% (w/v)) or Jeffamine (20% (w/v)), 0.2 M lithium or magnesium sulfate, at pH 5.5-7.5. A single crystal appeared with 0.1 M Bis-Tris pH 6.5, 0.2 M ammonium sulfate, 25% (w/v) PEG 3350. Before data collection, crystals were cryoprotected by brief immersion in precipitant solution containing 5% (v/v) glycerol. All crystals were cryocooled by plunging in liquid nitrogen and were stored under cryogenic conditions until data collection. A complete X-ray diffraction data set to 3.5-4.0 Å resolution was collected from single cryocooled (100 K) crystal at Alba MX beam line (Barcelona, Spain). Data was processed with XDS (Kabsch, 2010) and scaled with SCALA (Evans, 2006). The structure was phased by molecular replacement using Phaser (Mccoy, 2007) within the CCP4 suite (Winn *et al*., 2011).

5.2.10 Electrophoresis and immunobloting. One-dimensional, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as previously described (Almeida *et al*., 2014). For the dot-blot, 2µL of each sample were applied onto a nitrocellulose membrane (Roche, Mannheim, Germany). Membranes were blocked with TBST (50 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20) supplemented with 5% (w/v) skimmed milk, for 1 h at room temperature. Membranes were incubated with the anti-His antibody (Genescript) diluted 1:2000 in buffer TBSTM (TBST, 0.5% (w/v) skimmed milk) for 1 h, at room temperature and, after washing, incubated with the goat anti-mouse second antibody, diluted

1:10000 in the same buffer (GE Healthcare, Buckinghamshire, UK). Detection was performed by degradation of the ECF substrate (GE Healthcare) followed by development on a FX apparatus (BioRad, Hercules, EUA).

5.3 Results and Discussion

5.3.1 Cloning of Blad cDNA and its variant constructs into different expression vectors.

To maximize the chances of obtaining a recombinant soluble protein, without jeopardizing its biological activity, several alterations to the Blad amino acid sequence were introduced. This has already been described by Wu and Kim (Wu and Kim, 1997) for other proteins, where they substituted all of the hydrophobic amino acids in the helical domain of α-lactalbumine with leucine, obtaining a molecule that retains many features consistent with a native-like fold.

First, a preliminary bioinformatics analysis was performed using the primary sequence of Blad (Accession number: ABB13526, Monteiro *et al*., 2010) as template to predict its three-dimensional structure and analyze its spatial arrangement (Fig. 5.1). A total of six Blad-based clones (Fig. 5.2) were designed and inserted into four expression vectors of the pCoofy system.

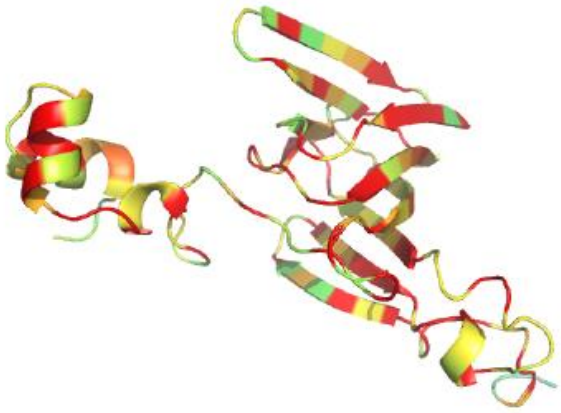

Figure 5.1. Predicted three dimensional structure of Blad using the the PHYRE 2 software.

Previous published works with β-conglycinin from soya (homologous to β-conglutin from lupins, Blad precursor) revealed that different variants of this polypeptide, with shortened N- and C- extremities, exhibited highly different solubility patterns, when compared with its native counterpart, proving that these regions might be relevant for solubility (Maruyama *et al*., 1999; Prak *et al*., 2007; Tandang-silvas *et al*., 2011). In addition to the original 173 amino acid sequence of Blad (Fig. 5.2, full length (FL) Blad – B1), five other constructs were designed to try to overcome the current insolubility issues: FL Blad with a mutation at residue 133 (Fig. 5.2, B2) – phenylalanine to tyrosine – that could improve solubility because phenylalanine is fully exposed to the solvent; Blad with 169 amino acid residues with a point mutation at residue 169

(Fig. 5.2, B3) – leucine to asparagine – that should improve packing between the C-terminal helix and the rest of the structure; a 136 residue Blad with a mutation on the amino acid 133 (Fig. 5.2, B4), like the B2 construct, but smaller in size; and an even smaller sequence of Blad with 125 residues and with a mutation on amino acid 100 (Fig. 5.2, B5) – alanine to valine – that should improve packing and optimize stability, particularly in shorter constructs. The C-terminal alone was also tested for expression and solubility giving that it is the expected portion of the sequence that could be causing the insolubility problems (Fig. 5.2, B6).

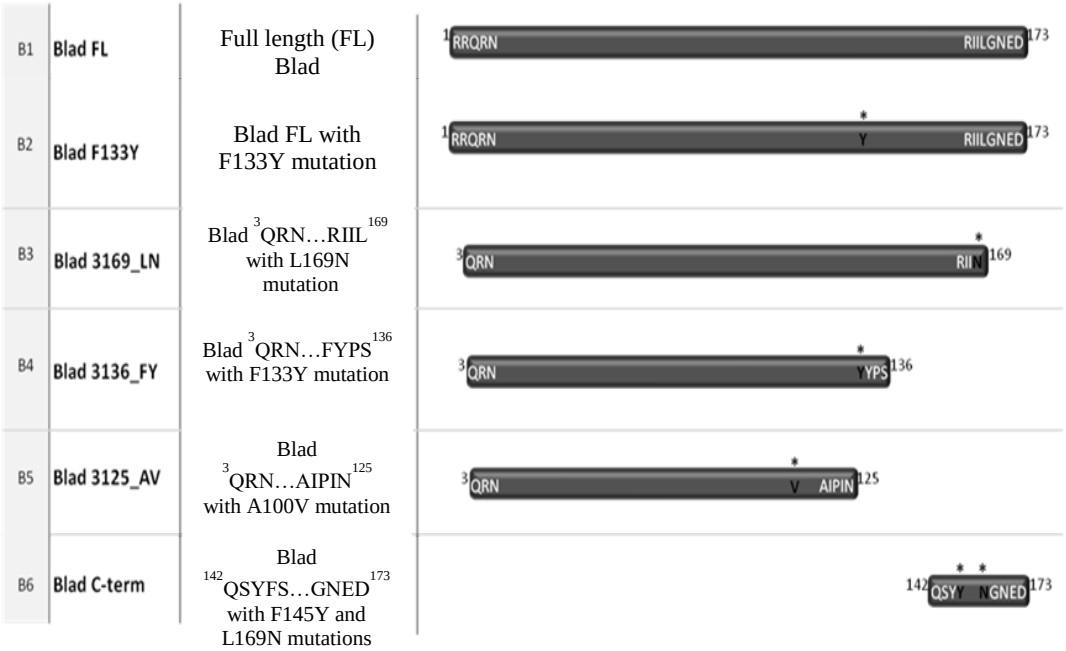

Figure 5.2. Schematic representation of Blad variant constructs designed based on the Blad primary sequence.

5.3.2 Screening of the optimal expression conditions. Each of the previously designed constructs was cloned together with four different fusion partners (His_6, His_6-MBP, Trx-His_6 and His_{10}-NusA). The screening was designed taking into account these 24 fusion proteins obtained previously (six Blad constructs in combination with four fusion partners), in combination with three different *E. coli* strains (BL21 Star™ (DE3), C41 (DE3) and Rosetta™ (DE3) pLysS), two culture media (LB and TB) and one set of expression parameters: induction of expression with 0.5 mM IPTG, for 6 h at 37 °C. Considering the proven antifungal activity of Blad (Monteiro *et al*., 2015; Pinheiro *et al*., 2016), an expression system using yeast as host was not an option.

This approach allowed the search for different heterologous expression conditions, which is a valuable tool for determining the optimal conditions to produce the target protein. After induction of expression for 6 h at 37 °C, cells were burst and both the total and the soluble fraction were initially analyzed through dot-blotting using an anti-Histag antibody (Fig. 5.3).

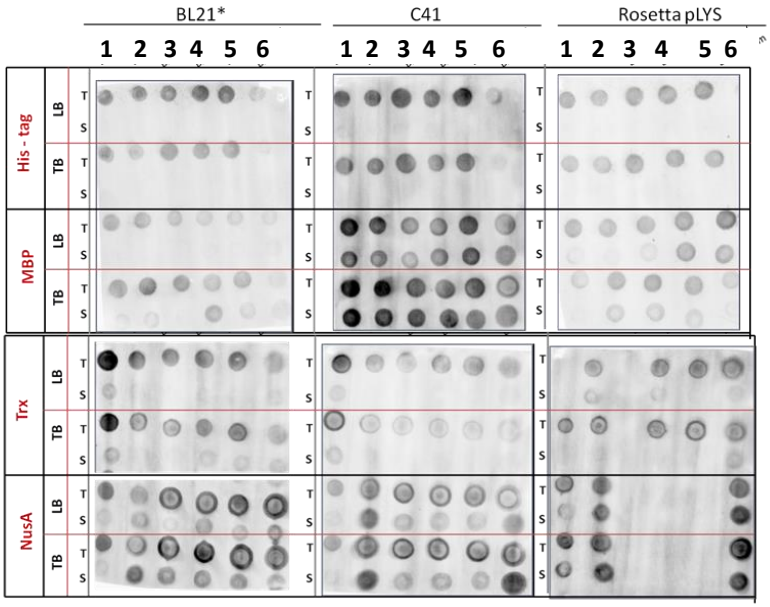

Figure 5.3. Analysis of the expression of Blad and its variant constructs. Proteins were expressed with the fusion partners His_6, His_6-MBP, Trx-His_6 or His_{10}-NusA, using three bacterial strains (BL21*, C41 or Rosetta pLyS) with two different media (LB and TB). The expression of the proteins was evaluated by dot-blotting, using an anti-Histag antibody as probe. Both the total (**T**) and the soluble (**S**) fractions were analyzed. Lanes **1**- B1; **2**- B2; **3**- B3; **4**- B4; **5**- B5 and **6**- B6.

The analysis of the total fraction (**T**) is only indicative of the level of expression of the target recombinant protein. Overall, all the constructs presented positive results on the dot-blot, with the constructs being expressed in the C41 cells presenting a higher amount of recombinant protein, followed by the BL21*. However, to perform a more accurate study it is important to also analyze the soluble fraction (**S**) giving that the target protein might be expressed but not in the desired soluble form. This was the case of the insoluble constructs that were expressed with only a polyhistidine tail as affinity tag (His_6). Despite the apparent good results obtained when looking at the total fraction, the analysis of the soluble fraction shows that all the His-tagged protein was being expressed as inclusion bodies, regardless the media culture, the Blad construct and the bacterial strain used. These results also corroborate the previous failed attempts to produce a soluble recombinant Blad without resorting to any solubility enhancing tag. Moreover, this result is indicative that the modifications imposed to the amino acid

sequence of Blad alone, whether it is cuts or point mutations, are not enough to improve its solubility (Fig. 5.3 line 1).

When using a solubility enhancing tag as fusion partner, only the constructs with His_6-MBP and His_{10}-NusA resulted in the proper accumulation of the protein in a soluble form. Moreover, this result was more noticeable when the constructs were being expressed in C41 cells in TB medium. The presence of the Trx-His_6 as fusion partner did not cause any positive influence on the increase of the solubility of the various Blad constructs, regardless the bacterial strain and medium used.

A second analysis was performed with only the soluble fraction of each construct, obtained after over-expression under the most promising condition: C41 cells in TB medium, for 6 h at 37 °C. The dot-blot is a satisfactory first indicator of the presence of the target protein. However, the target protein might be expressed in soluble form but without the expected molecular weight, due to degradation phenomena. A SDS-PAGE was performed to assess if each construct is being expressed not only in a soluble form but also with the expected molecular weight (Fig. 5.4). The expected molecular weight of each construct, in combination with each one of the fusion partners used, is shown in Table 1.

Table 5.1. Expected molecular weight of each Blad construct with each one of the fusion partners (kDa).

Construct	His_6 (0.84 kDa)	Trx-His_6 (12.2 kDa)	His_6-MBP (40 kDa)	His_{10}-NusA (56 kDa)
B1 (20.5 kDa)	21.3	32.7	60.5	76.5
B2 (20.5 kDa)	21.3	32.7	60.5	76.5
B3 (19.6 kDa)	20.4	31.8	59.6	75.6
B4 (15.7 kDa)	16.5	27.9	55.7	71.7
B5 (14.4 kDa)	15.2	26.6	54.4	70.4
B6 (4.5 kDa)	5.3	16.7	44.5	60.5

Analysis of Figure 4 confirms the previous results obtained with dot-blotting: the constructs fused with His_6 or Trx-His_6 did not translate in the accumulation of soluble recombinant protein with the expected molecular weight. Regarding the results obtained with His_6-MBP and His_{10}-NusA, His_6-MBP as a fusion partner is the most promising condition, regardless the Blad

construct, considering that it is where it is visible a superior accumulation of soluble protein with the expected molecular weight.

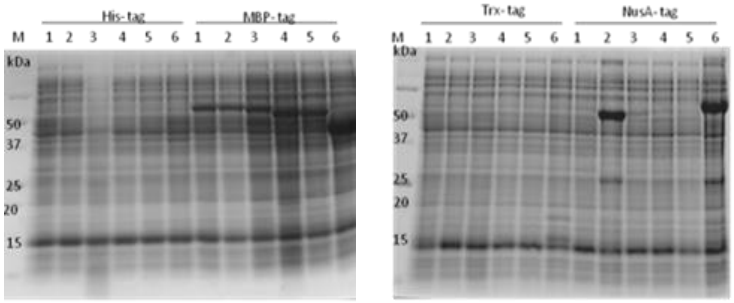

Figure 5.4. SDS-PAGE analysis of the soluble fraction of each construct after induction of expression in C41 *E. coli* cells, in TB medium in a 12% (w/v) acrylamide gel, stained with Coomassie Brilliant Blue R (Sigma). Lanes **1**- B1; **2**- B2; **3**- B3; **4**- B4; **5**- B5 and **6**- B6. Molecular masses of standards are indicated in kDa

The soluble fraction of the six Blad-based constructs expressed with His_6-MBP as fusion partner were subjected to western blotting analysis, to confirm the proper expression of the protein. Figure 5.5 shows that all Blad constructs were correctly expressed, presenting the expected molecular weight. Considering that the five novel Blad-based constructs designed did not seem to enhance Blad solubility, the work continued only with the original sequence of Blad (i.e. B1).

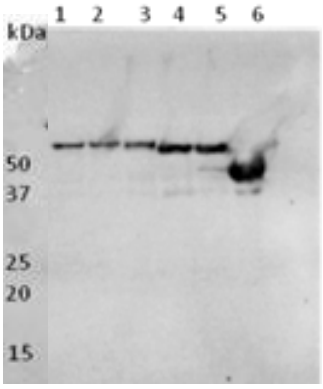

Figure 5.5. Immunodetection of Blad with His_6-MBP as fusion partner, using an antibody anti-Histag as probe. Lanes **1**- B1; **2**- B2; **3**- B3; **4**- B4; **5**- B5 and **6**- B6. Molecular masses of standards are indicated in kDa

5.3.3 Purification of the target fusion protein. After screening and establishing the optimal expression conditions, it was necessary to scale-up to purify the target protein with good yields and suitable quantities. Considering that the best solubility-enhancing partner is the His_6-MBP, two different purification strategies could be applied: one using an affinity column for the polyHistidine tail (HisTrap), another directed to MBP (MBPtrap). Comparing the results obtained with both strategies, the target protein was obtained with a higher purity level when using a MBPtrap, a column charged with dextrin to which MBP has affinity (data not shown).

The soluble fraction obtained after expression of full length Blad with His_6-MBP (His_6-MBP-Blad) in C41 bacterial cells in TB medium, the best set of conditions chosen in the previous screenings, was applied to a MBPtrap column, washed and eluted with 10 mM maltose in binding buffer. The results are presented in Figure 5.6A. The eluted fraction was further evaluated by SDS-PAGE (Fig. 5.6B). Purification of His_6-MBP-Blad in a MBPTrap resulted in the elution of the fusion protein with a high purity level, as shown by both the chromatogram and the SDS-PAGE. (Fig. 5.6).

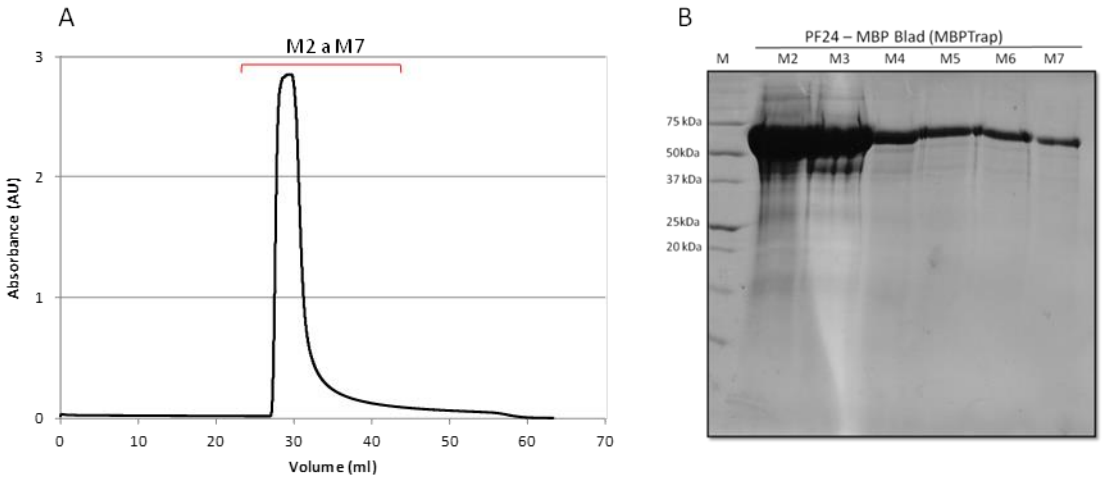

Figure 5.6. Purification of the His_6-MBP-Blad by affinity chromatography using a MBPtrap column. The bound protein was eluted with 10 mM maltose in binding buffer (**A**). SDS-PAGE analysis of the eluted fractions (M2 to M7) in 12% (w/v) acrylamide gel, stained with Coomassie Brilliant Blue R (Sigma) (**B**). Molecular masses of standards are indicated in kDa

In the following set of experiments, the oligomerization status of the purified recombinant protein was analyzed through molecular exclusion chromatography, with a Superose 6 (10/300

GL) column. The exclusion volume of the column was determined by the elution of blue dextran (average molecular weight of 2 MDa; eluted at 8.5 mL) and thyroglobulin (600 kDa; eluted at 12.85 mL). Fractions M2, M3 and M4 eluted from the MBPtrap (Fig. 5.6B) were loaded onto a Superose 6 column and eluted with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol. As shown in Figure 5.7, the eluted fraction of MBPTrap consists of four His_6-MBP-Blad oligomers: one eluting at 8 mL (a), corresponding to large protein aggregates. According to the column specifications, all protein species with a molecular weight superior to the column resolution capacity are eluted at 8 mL. This result was further confirmed by SDS-PAGE, where a single band at 60 kDa was visible, confirming that it was not a contaminant (data not shown); one eluting at 12 mL (b) and corresponding to oligomers of the fusion protein with approximately 600 kDa. This result was confirmed by the presence of a single 60 kDa band on SDS-PAGE (data not shown) and by the posterior structural characterization studies (results below); one species (c) corresponding to the isolated MBP, as confirmed by the elution volume of approximately 18 mL (according to this column specifications, it corresponds to a protein with a molecular weight similar to that of MBP) and by a single 40 kDa band on SDS-PAGE (data not shown); and finally the non-oligomeric globular species of the recombinant protein (d). This fraction was analyzed by SDS-PAGE and revealed a surprising molecular weight of 60 kDa band (data not shown). If it was folded in a structurally correct form, it should have been eluted before isolated MBP. If a protein is eluted much later than it was supposed, it is possible that it is folded in an unusual form.

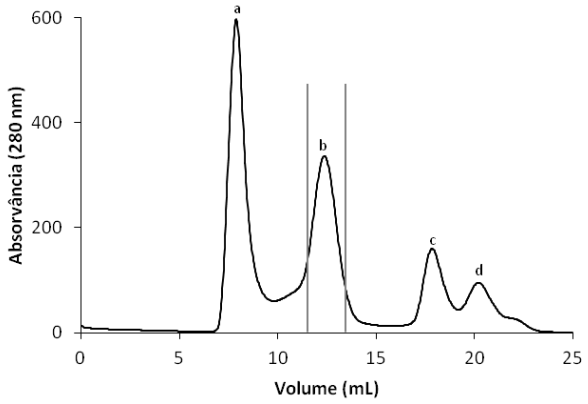

Figure 5.7. Purification of the His_6-MBP-Blad by gel filtration chromatography on a Superose 6 10/300 GL column. Elution was with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol.

5.3.4 Preliminary structural characterization of the His6-MBP-Blad oligomers.

The previously obtained His_6-MBP-Blad oligomers were analyzed by DLS and TEM, a quality control commonly used to characterize the homogeneity and structure of a given sample.

The results obtained by DLS (Fig. 5.8) indicate that the purified fusion protein is mostly composed by high molecular mass oligomers, as indicated by its polydispersity index (PdI=0.226). His_6-MBP-Blad oligomers presented a hydrodynamic radius of 9.6 nm, which corresponds to a putative molecular weight of 663 kDa. This result is in accordance with the estimated molecular weight obtained after purification in the Superose 6 10/300 GL column (Fig. 5.7, peak b).

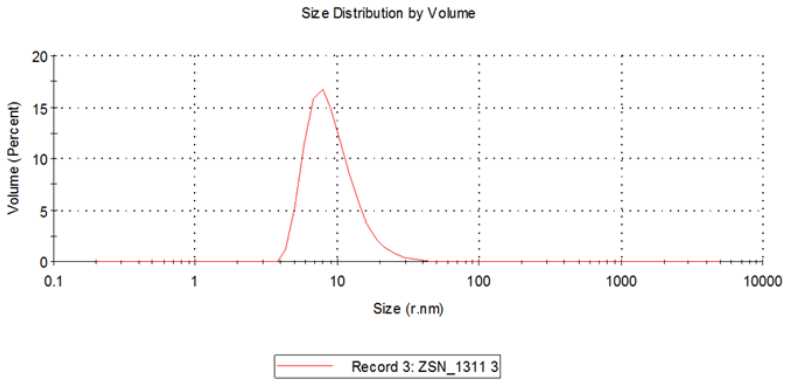

Figure 5.8. DLS profile of His_6-MBP-Blad oligomers. r.nm: radius, expressed in nm.

His_6-MBP-Blad oligomers were also analyzed by negative-staining electron microscopy, which revealed that the fusion protein seems to be composed of two (possibly heptameric) rings, with each ring having an approximate diameter of 16 nm (Fig. 5.9). These preliminary studies of structural characterization revealed that these oligomers are structurally homogeneous and therefore, are a good fit for the posteriors structural analysis.

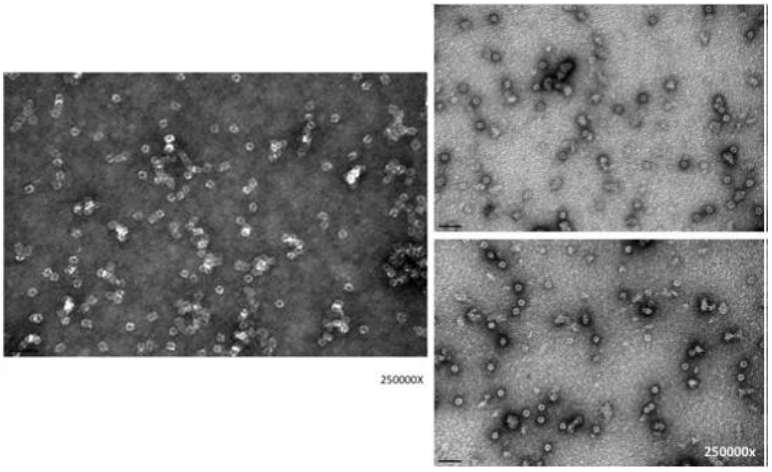

Figure 5.9. Electronic microscopy images of His_6-Blad-MBP oligomers obtained by negative staining TEM.

5.3.5 Removal of the fusion partner. Once the target fusion protein was purified with good yields and in a structurally homogeneous form, the next step would be the removal of the solubility-enhancer partner. One of the most used methods for affinity tag removal includes enzymatic cleavage of the tag followed by specific removal of the processing enzyme by an affinity chromatographic step to yield the detagged pure protein (Arnau *et al*., 2006). As described in the materials and methods section, the constructions used in this study were designed using a cleavage site between the solubility-enhancing partner and the target protein. The 3C protease, an enzyme designed to remove purification tags from the protein of interest, is highly specific for the recognition sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro and cleaves after the glutamine residue.

Figure 5.10 shows the results obtained after treatment of the purified His_6-MBP-Blad oligomers with the 3C protease. After incubation, both soluble and insoluble fractions were analyzed by SDS-PAGE. In the soluble fraction, a 40 kDa band corresponding to MBP and a 60 kDa band corresponding to remains of the construct that were not digested by the 3C protease are clearly visible (Fig. 5.10, lane "sol"). The 20 kDa band corresponding to Blad is predominantly present in the insoluble fraction (Fig. 5.10, lane "Ins"). Even after several attempts of digestion with the 3C protease, where different temperatures of incubation and additives were tested, recombinant free Blad was always insoluble (data not shown). This is in agreement with the literature, which claims that after tag removal, the final solubility of the desire product is

unpredictable (Rosano and Ceccarelli, 2014). In general, whereas folded proteins tend to remain soluble, incompletely or improperly folded proteins precipitate when they are cleaved from MBP (Nallamsetty and Waugh, 2006). Nevertheless, the resolution of the three-dimensional structure of proteins containing large-affinity tags is well documented (Ke and Wolberger, 2003; Kobe *et al*., 1999; Liu *et al*., 2001) and thence the posterior assays were performed with the Blad fused to MBP (His_6-MBP-Blad).

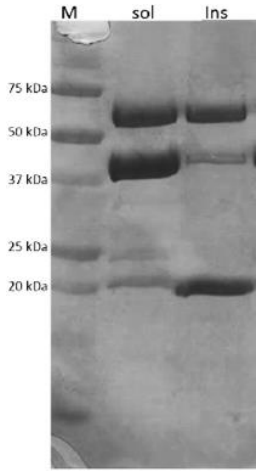

Figure 5.10 SDS-PAGE analysis (12% (w/v) acrylamide gel, stained with Coomassie Brilliant Blue R) of the soluble (**sol**) and insoluble (**ins**) fractions obtained after incubating the His_6-Blad-MBP oligomers with the 3C protease. Molecular masses of standards are indicated in kDa

5.3.6 Characterization of the three-dimensional structure of His6-MBP-Blad oligomers. The oligomers of His_6-MBP-Blad were further analyzed by SAXS to confirm and obtain accurate information about their global form and oligomerization status in solution (Fig. 5.11). The His_6-MBP-Blad scattering profile (Fig. 5.11a) was derived after subtracting the averaged buffer scattering patterns, normalizing by each concentration and extrapolating to infinite dilution with PRIMUS (Konarev *et al*., 2003). The Guinier radius of gyration (Rg) (Figure 5.11a, inset) remained constant within the experimental errors in the range of concentrations measured (Rg = 66 Å ± 1 Å and Rg = 68 Å ± 1 Å for 1 mg/mL and 2 mg/mL, respectively), indicating minimal contribution of interparticle effects. The pair-distance distribution function, *P*(r), calculated from the scattering data, reached a maximum value at 89 Å and extended to maximum distances (*D*max) of about 204 Å (Figure 5.11b). This *P*(r) bell-shaped curve is characteristic of globular and spherical particles. The high apparent molecular weight

estimated from the Porod volume was of 1.15 MDa, suggesting formation of an oligomeric arrangement since the theoretical MW calculated from the sequence is approximately 60 kDa. The *ab initio* shape reconstructions were calculated with DAMMIF (Franke and Svergun, 2009). Fifteen independent models were generated. The set of structures were superimposed and an averaged model that represents the most populated volume was calculated with DAMAVER (Volkov and Svergun, 2003). A normalized spatial discrepancy (NSD) of 0.79 was obtained, indicating a low structural variability between the 15 independent *ab-initio* models. For representation, volumetric maps were calculated from the bead models with the SITUS package (Wriggers, 2010). The averaged model is shown in Figure 5.12.

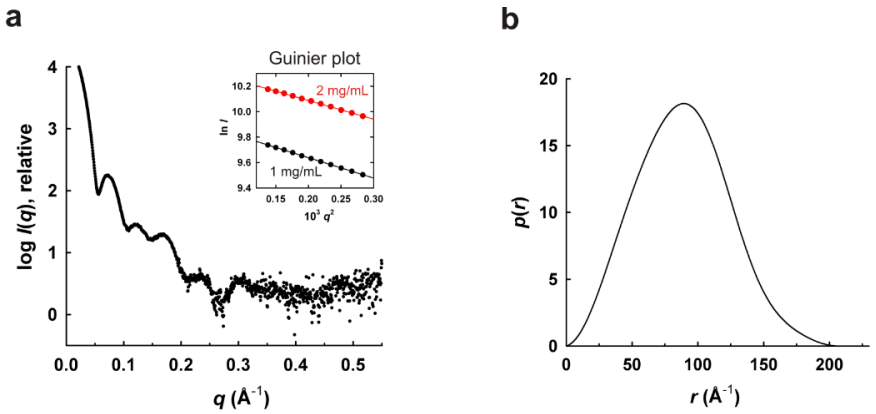

Figure 5.11. SAXS analysis of His_6-MBP-Blad oligomers in solution. **a**) SAXS profile extrapolated to infinite dilution. Curve is offset on the log scale. The Guinier plot at several protein concentrations and the linear regression fit are shown in the *inset*. **b**) *P(r)* function determined from the scattering data.

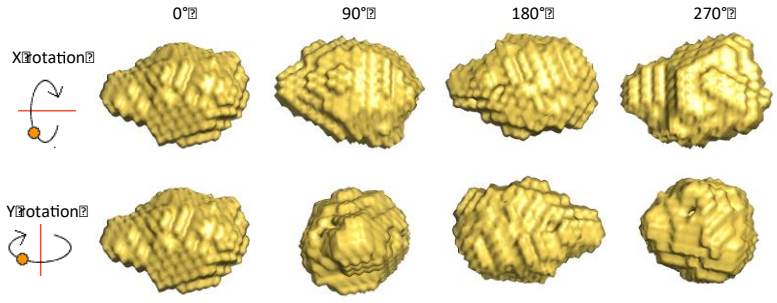

Figure 5.12. SAXS-derived molecular envelopes (yellow-solid surfaces). The low-resolution structures are the average of 15 independent bead models. Four orthogonal views along the X and Y axes are shown.

5.3.7 Crystallization, data collection and structure solution. The recombinant fusion protein His_6-MBP-Blad was subjected to different crystallization conditions, leading to the appearance of crystals after 2-3 weeks (Fig. 5.13), which were tested for X-ray diffraction analysis. The structure of the fusion protein was solved with a maximum resolution of 3.5 Å, revealing that the crystals belong to the triclinic spatial group P1 with a unit cell a=135.06 Å, b=188.67 Å, c=261.40 Å, α=98.93°, β= 90.74° and γ=104.89°.

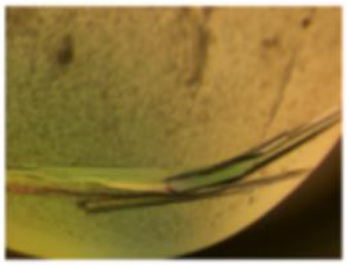

Figure 5.13. Crystals of His_6-MBP-Blad obtained by X-ray diffraction data.

After applying the molecular replacement technique, using the tridimensional structure of MBP and a theoretical model of Blad, it was not possible to obtain enough X-ray diffraction data to resolve the tridimensional structure of the construct. Another approach was to use a tridimensional model of a heptameric protein with a molecular mass similar to that of His_6-MBP-Blad. Surprisingly, the refinement and optimization of the model revealed the presence of two molecules of the bacterial chaperonine GroEL (Fei *et al*., 2013) in each asymmetric unit of the crystal. The three dimensional structure of GroEL has been described as a porous cylinder of 14 subunits made of two 7-fold symmetrical rings (Braig *et al*., 1994). Figure 5.14 shows the perfect overlapping of the electron density maps obtained by X-ray diffraction of the His_6-MBP-Blad crystals with the three dimensional structure of the chaperonine GroEL from *E. coli*, leaving no doubts about the nature of the crystals.

Host chaperone protein is a common contaminant that is often co-purified with tagged proteins and its co-purification has been extensively discussed (Farkas *et al*., 2006; Keresztessy *et al*., 1996; Marco *et al*., 2004; Rohman and Harrison-Lavoie, 2000). Unfortunately, considering that the molecular mass of GroEL is practically the same as that of His_6-MBP-Blad (approximately 60 kDa), it was impossible to detect the contamination on any SDS-PAGE. In fact, in Figure 5.10,

a 60 kDa band remains visible after treatment with the 3C protease, when only a 40 kDa and a 20 kDa bands, corresponding to MBP and Blad, respectively, were supposed to be present. The presence of that 60 kDa band may have been misinterpreted as the remains of the construct that were not digested by the protease.

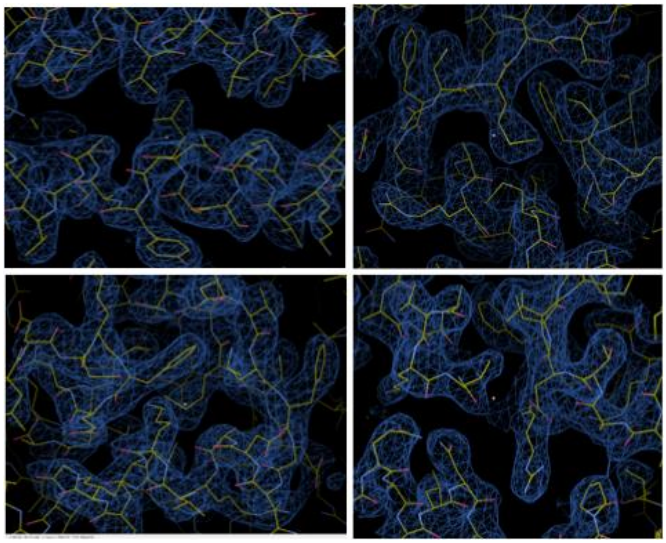

Figure 5.14. Electron density map (experimental result obtained by X-ray diffraction of the crystals of His_6-MBP-Blad overlapped with the three dimensional structure of the chaperonine GroEL from E. coli.

5.3.8 Release of GroEL from the fusion protein. Rial and colleagues (Rial and Ceccarelli, 2002) reported an easy approach to eliminate contaminations with host chaperone proteins by washing the fusion protein with MgATP. After a 30 min incubation with 5 mM MgATP at room temperature, the complex GroEL-His_6-MBP-Blad was loaded onto a chelating Sepharose column, washed and eluted with 20 mM Tris-HCl pH 8.0, 0.1 M NaCl and 0.5 M imidazole. All fractions were analyzed by SDS-PAGE (Fig. 5.15A). Considering the similarity of molecular masses between GroEL and His_6-MBP-Blad, the expected 60 kDa band obtained in the eluted fraction is not clarifying *per si*. The eluted fraction was posteriorly incubated with the 3C protease and both the soluble and the insoluble fractions were analyzed by SDS-PAGE (Fig. 5.15B). If the incubation with MgATP was effective, we would expect to see two bands, one with 40 kDa corresponding to MBP and one with 20 kDa corresponding to Blad. The results obtained suggest that the incubation with MgATP was not enough to separate the GroEL from the fusion protein since a 60 kDa band is still present. Moreover, Blad only appears on the insoluble fraction meaning that after these treatments Blad turns insoluble, even after co-expression with

MBP. The same experiment was repeated after incubation with 10 mM MgATP for both 4 h and overnight but the contamination with GroEL persisted (data not shown).

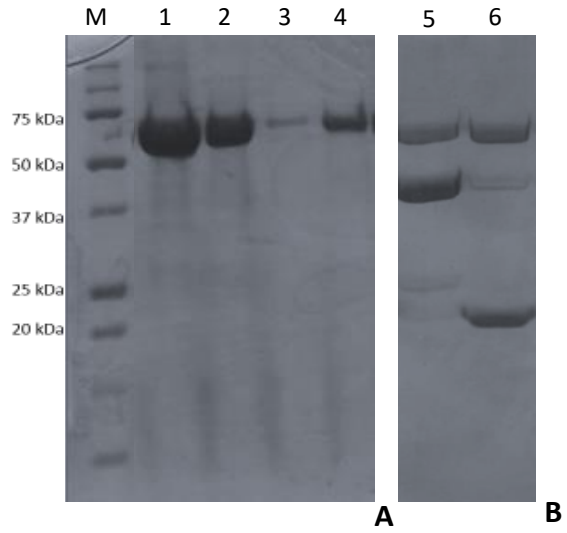

Figure 5.15. SDS-PAGE analysis in a 12% (w/v) acrylamide gel, stained with Coomassie Brilliant Blue R (Sigma) of the: (**A**) complex GroEL-His_6-MBP-Blad after incubation with 5 mM MgATP (**1**) and posterior incubation in a chelating Sepharose resin: flow-through (**2**), wash (**3**) and eluted fraction (**4**); and (**B**) the resulting soluble (**5**) and insoluble (**6**) fractions of the eluted fraction after incubation with the 3C protease. Molecular masses of standards are indicated in kDa

Considering the latest results, the screening approach used led to the production of the fusion protein linked to GroEL, an *E. coli* chaperone contaminant. In the future, and giving the impossibility to separate the host chaperone from the fusion protein, a different approach must be followed to allow the production of the recombinant Blad, in a soluble form, and without host protein contamination.

5.4 Supplementary Material

Supplementary Table S5.1. Plasmids used in this work.

Vector	N-tag	Reference
pCoofy 1	His 6	Scholz *et al.*, 2013
pCoofy 2	Trx-His6	
pCoofy 4	His6-MBP	
pCoofy 16	His10-NusA	

Supplementary Table S5.2. Oligonucleotides used in this work.

Construct	Oligonucleotide	Sequence (5' -> 3')
Blad FL	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCGTCGTCAGCGTAATCCG
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**ATCTTCGTTGCCCAGAATAATG
Blad 3169_LN	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCAGCGTAATCCGTATCATTTTAGCAG
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**GTTAATAATGCGCTGAATTTCTTCATAGC
Blad F133Y	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCGTCGTCAGCGTAATCCG
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**ATCTTCGTTGCCCAGAATAATG
Blad 3136_FY	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCAGCGTAATCCGTATCATTTTAGCAG
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**GCTCGGGTA***ata***ATCATAGAAATAACC
Blad 3125_AV	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCAGCGTAATCCGTATCATTTTAGCAG
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**ATTGATCGGAATGGCCAGTTTAAC
Blad C-term	Forward	GCGGGTCTGGAAGTTCTGTTCCAGGGGCCCCAGAGCTATTATAGCGGTTTTAGCC
	Reverse	TATATTCCCCAGAACATCAGGTTAATGGCG**TTA**ATCTTCGTTGCC***gtt***AATAATGC

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Chapter 6

Fusion proteins towards fungi and bacterial in plant protection

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Chapter 6 - Fusion proteins towards fungi and bacteria in plant protection

Abstract

In agriculture, although fungi are considered a foremost problem, infections by bacteria also cause significant economical losses. The presence of different diseases in the crops often leads to a misuse of the proper therapeutic or the combination of different diseases forces the use of more than one pesticide. This work aimed at the development of a "super-Blad": a chimeric protein consisting of Blad polypeptide, the active ingredient of a biological fungicide already in the market, and two selected peptides, SP10-5 and Sub5, proved to possess biological potential as antibacterial agents.

The resulting chimeric protein obtained from the fusion of Blad with SP10-5 not only maintained a strong antibacterial activity, especially against *Xanthomonas* spp. and *Pseudomonas syringae,* but was also able to retain the ability to inhibit the growth of both yeast and filamentous fungi. However, the antibacterial activity of Sub5 was considerably diminished when fused to Blad, which seems to indicate that not all fusion proteins behave equally.

These designed drugs can be considered promising compounds for use in plant protection. A deeper and focused development of an appropriate formulation may result in a potent biopesticide which can replace *per se* two conventional chemistries with less impact on the environment.

Keywords: biopesticides, biotechnology, antimicrobial peptides, Blad-containing Oligomer, pest management

6.1 Introduction

The quality and yield of crop production have been adversely affected by a large variety of pests, like bacteria, fungi, weeds and insects (Kumar and Singh, 2015). Despite the undeniable role posed by fungi and viruses as the most important plant pathogens, infections by bacterial are rising and becoming more severe (Eichenlaub *et al*., 2007), making them a major threat to agriculture due to the lack of suitable agrochemicals and the absence of resistance and/or immunity in host plants. Moreover, they are usually spread undetected as contaminants or asymptomatic (latent) infections in plant propagation materials (van der Wolf and De Boer, 2015). Another problem is the common error when identifying a plant disease. The manual identification is a rather subjective task and laboratory analyses are often time consuming, leading to an unacceptable lag between observation and identification of the disease. Besides, the symptoms of some diseases are highly heterogeneous, making them difficult to identify. Moreover, the simultaneous presence of different diseases may exhibit a combination of symptoms quite different from the each disease *per se* (Garcia and Barbedo, 2016). As a whole, these factors often lead to a misinterpretation of the disease which ultimately primes to a misuse of a proper therapeutic or the combination of different diseases forces the use of more than one pesticide.

The abuse and incorrect use of agrochemicals has driven to pesticide resistance, pest resurgence, outbreak of secondary pests, pesticide residues in the produce, soil, air and water (Al-Zaidi *et al*., 2011). Biopesticides are becoming more appealing because of their advantages in terms of effectiveness, environmental safety, specificity, biodegradability and suitability in the integrated pest management (IPM) programs. Thus, biopesticides are promising alternatives to manage environmental pollutions. The use of the new generation of microbial biopesticides has registered a steady increase of 10% every year, and more than 225 microbial biopesticides are registered in 30 Organization for Economic Co-Operation and Development (OECD) countries (Hubbard *et al*., 2014). Nevertheless, biopesticides are still a young and evolving science where a profound research is still needed from production and formulation to delivery and commercialization of these products.

For the past few years, antimicrobial peptides have become a promising therapeutic both in medicine and in agriculture considering their relatively small size (easy to synthesize), their fastness and efficiency, their antimicrobial activity against a wide range of pathogens and their low toxicity for vertebrate cells (Bang *et al*., 2012; Hancock *et al*., 2006). However, some studies

demonstrate that the simultaneous use of more than one antimicrobial peptide is usually a better approach to increase its effectiveness in plant protection or for pharmaceutical uses. To achieve this, a simple strategy is to fuse together two protein sequences with the same specialized (Hongbiao *et al*., 2005), or complementary functions (Kovalskaya *et al*., 2011; Lee *et al*., 2013), originating a novel protein called a fusion, or chimeric protein (Ingham and Moore, 2007).

Blad is the active ingredient of a biological fungicide already in the market discovered by us. It is a 20.4 kDa polypeptide, being the major subunit of a 210 kDa glyco-oligomer, termed Blad-containing oligomer (BCO), which accumulates in *Lupinus albus* cotyledons between days 4 and 12 after the onset of germination. The characteristics that makes the BCO a unique, versatile and multifunctional protein (Monteiro *et al*., 2015) as well as its powerful and broad spectrum antifungal activity towards both plant and human pathogens were already fully discussed (Monteiro *et al*., 2015; Pinheiro *et al*., 2016). Its mode of action was also recently described as a multi-site fungicide that disturbs microbial cell homeostasis leading, ultimately, to cell death (Pinheiro *et al*., 2017).

In this work, we report the development of a "super-Blad" – a fusion gene consisting of the gene that codes for Blad and genes that code for selected peptides proved to possess biological potential as antibacterial agents. SP10-5 is a 12 amino acid peptide experimentally designed by Zeitler and his colleagues using the scorpion-derived antimicrobial peptide IsCT and the frog-derived peptide mangainin II as templates (Zeitler *et al*., 2013). SP10-5 was tested against several bacteria and presented promising results (Zeitler *et al*., 2013). Sub5 is a 12 amino acid cationic peptide synthetized from a linearized variant of the bovine peptide bactenecin, Bac2A, that acts on the external membrane of Gram negative bacteria (Hilpert *et al*., 2005). Sub5 presents not only antibacterial activity against an array of both gram-positive and gram-negative bacteria but also antifungal activity against *Candida albicans* (Ebbensgaard *et al*., 2015; Hilpert *et al*., 2005).

The current investigation is in line with the new approach to produce novel fusion proteins with improved antimicrobial performance, which may represent the future of the technological development of biopesticides. Here we report the fusion of two proteins with distinct

antimicrobial properties, one fungicidal and the other bactericidal, creating a new "super-molecule" that enables to treat simultaneously fungal and bacterial infections in plants.

6.2 Materials and Methods

6.2.1 Strains and media. DH5α™ competent *Escherichia coli* cells were used for all routine cloning experiments. For recombinant protein overexpression, C41 (DE3) *E. coli* strain was used, cultivated in TB (12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4 and 4 mL/L glycerol) medium.

Strains of *E. coli, Salmonella thypimurium, Pseudomonas aeruginosa* and *Staphylococcus aureus* were a courtesy of Instituto Superior de Agronomia (ISA-UL). Strains of *Xanthomonas arboricola* pv. *pruni* (NCPPB 2878 and NCPPB 3744), *X. versicatoria* (NCPPB 3801 and NCPPB 3954) *and Pseudomonas syringae* pv. *tomato* (NCPPB 3645 and NCPPB 4369) were purchased from the National Collection of Plant Pathogenic Bacteria (NCPPB, UK). All strains were grown at 34°C for 24 h in Tryptone Soy Agar (TSA) medium (3% (w/v) Tryptone Soy Broth, 1.5% (w/v) Agar). For the antibacterial susceptibility tests bacteria were grown in Mueller-Hinton medium.

C. albicans var. *albicans* (CBS 562) [CBS- Centraalbureau voor Schimmelcultures] and *C. glabrata* (a kind gift from of Institute of Microbiology, Faculty of Medicine of the University of Coimbra (Paulo *et al*., 2009)) were grown at 34°C for 24 h in Glucose Yeast Peptone (GYP) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar).

Botrytis cinearea was isolated in our lab (from tomato) and was grown on Sabouraud Dextrose Agar for 7 days at 25°C. For the antifungal susceptibility tests the medium used was PDB medium (2.4% (w/v) Potato Dextrose Broth), buffered at pH 7.5.

6.2.2 Plant material. *Lupinus albus* L. seeds were purchased from Inveja SAS (France) and were germinated as described by Pinheiro and colleagues (Pinheiro *et al*., 2017).

6.2.3 Production of BCO. Blad-containing oligomer (BCO) was extracted and purified from 8-days-old cotyledons as previously described (Monteiro *et al*., 2010) and stored lyophilized at room temperature

6.2.4 Design of synthetic genes and cloning procedures. Synthetic genes were purchased from GeneScript, Hong Kong Limited and cloned into pCoofy plasmids, a bacterial expression vector containing N-terminal 6x His and MBP tags (Scholz *et al*., 2013). General cloning procedures were performed as described by Scholz and colleagues (Singh *et al*., 2015). The correct nucleotide sequence of the inserts in all the constructed plasmids was checked by DNA sequencing.

6.2.5 Expression of the fusion proteins. Cells harboring plasmids encoding the different fusion proteins were grown overnight at 37 °C with shaking, in TB medium supplemented with kanamycin. For the initial expression screening, 20 µL of the preculture were inoculated in 2 mL of fresh media on a 24-well plate. For production upscaling, 10 mL of the preculture were used to inoculate 1 L of fresh media. Inoculates were grown at different temperatures (20, 25, 30 and 37 °C) until $OD_{600\ nm}$ reached 0.4 and then induced with three different concentrations of IPTG (Isopropyl β-D-1-thiogalactopyranoside) (0.1, 0.5 and 1 mM). The cells were pelleted after 6 h by centrifuged at 4000 *g,* 4 °C, for 20 min.

6.2.6 Fusion protein purification. Cell pellets were ressuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.25 mg/mL lysozyme), submitted to a freezing/thaw cycle and then incubated with DNase (2.5 µg/mL) and $MgCl_2$ (5 mM), thus originating the total fraction. The soluble and insoluble fractions were separated by centrifugation (3500 *g*, 10 min, 4 °C).

For purification purposes, a MBPTrap HP column (GE Healthcare) was used, previously equilibrated with 20 mM Tris-HCl pH 8.0, 2 mM Ethylenediamine tetraacetic acid (EDTA), 150 mM NaCl and 10 % (v/v) glycerol, and eluted with 10 mM maltose in binding buffer.

6.2.7 Solubilization and refolding of inclusion bodies. The solubilization and refolding of inclusion bodies was performed as described by Singh and his colleagues (Singh *et al*., 2015). Briefly, the insoluble fraction resulting from the expression of the fusion proteins was ressuspended in wash buffer A (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride (PMSF)) and centrifuged at 20,000 *g* for 20 mins at 4 °C. The pellet was ressuspended in wash buffer B (50 mM Tris-HCl pH 8.5) and centrifuged again in the same conditions. The final pellet was ressuspended in milliQ water and kept frozen at -80 °C

until used. For solubilization purposes, 5 mg of inclusion bodies were suspended in solubilization buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM PMSF, 8 M urea), mixed by vortexing and incubated at room temperature for 1 h. The solubilized proteins were centrifuged at 20,000 *g* for 30 min at 4 °C. For the refolding, 1 mL of the supernatant was added in small amounts in regular intervals to 9 mL of freshly cooled refolding buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 % (w/v), 1 mM PMSF). The refolded sample was kept at 4 °C for 6 h and then filtered through a 0.45 µM polyvinylidene fluoride (PVDF) filter to remove protein aggregates.

6.2.8 Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis.

In gel digestion. The gel bands were sliced into small pieces, destained and incubated overnight with trypsin for protein digestion. After the digestion, peptides were extracted from the gel using 3 different solutions of increased percentage of organic solvent (water/acetonitrile with 1 % (v/v) formic acid). Peptides were ressuspended in 30 µL of a solution containing 2 % (v/v) acetonitrile and 0.1 % (v/v) formic acid and analyzed by LC-MS/MS.

LC-MS/MS analysis. Samples were analyzed on an AB Sciex 5600 TripleTOF (ABSciex®) in information-dependent acquisition (IDA) mode. Peptides were fractioned by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXPTM C18CL reverse phase column (300 µm ID × 15 cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5 µL/min and eluted into the mass spectrometer with an acetonitrile gradient in 0.1% FA (2% to 30% ACN, in a linear gradient for 30 min), using an electrospray ionization source (DuoSprayTM Source, ABSciex®) with a 50 µm internal diameter (ID) stainless steel emitter (New Objective). For information dependent acquisition (IDA) experiments the mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250ms, followed by up to 80 MS/MS scans (100–1500 m/z from a dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 2.7 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 15 seconds (mass spectrometer operated by Analyst® TF 1.6, ABSciex®). Rolling collision was used with a collision energy spread of 5.

Protein identification. Protein identification was obtained using Protein Pilot™ software (v 5.1, ABSciex) with the following parameters: search against the databased provided by (with the sequences of the recombinant proteins) and against the uniprot database from

June 2016, with acrylamide alkylation and trypsin digestion. Positive identification was considered for protein that met the 1.3 unused score value and 95% peptide confidence filtering.

6.2.9 Bacteria, yeasts and filamentous fungi inhibition tests. The susceptibility tests were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guidelines M27-A2, M31-A2 and M38-A2 for yeasts, bacteria and filamentous fungi, respectively (CLSI, 2008; NCCLS, 2002a, 2002b) with small adjustments.

Bacteria were grown on TSYA medium, overnight at 34°C. The inoculum suspension was then prepared in 5 mL of sterile 0.9% (w/v) saline solution (NaCl) and cell density was adjusted with a spectrophotometer, in order to obtain a concentration of $1x10^8$ cells/mL. The final inoculum suspension was made by a 1:100 dilution with double-strength Mueller-Hinton medium, in order to achieve a concentration of $1x10^6$ cells/mL. The inoculum size was confirmed by enumeration of CFU on TSYA plates.

The susceptibility tests made on yeasts and filamentous fungi were performed as previously described (Monteiro *et al*., 2015; Pinheiro *et al*., 2016).

In all cases, each well of the microplate, contained 100 µL of the inocula and 100 µL of the diluted drug solution (twofold).

The microplates were incubated at 34°C and 25 °C for yeasts and filamentous fungi, respectively, and examined after 72 h. Bacteria were incubated at 30°C and the results were taken after 24 h to 72 h, according to their growth rate. The MIC endpoints were recorded visually as the lowest drug concentration that showed absence of growth.

6.2.10 Electrophoresis and immunoblotting. One-dimensional, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as previously described (Monteiro *et al*., 2010). For the dot-blot, 2µL of each sample were applied to a nitrocellulose membrane (Roche, Mannheim, Germany). Membranes were blocked with TBST (50 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20) supplemented with 5% (w/v) skim milk, for 1 h at room temperature. Membranes were incubated with the antibody anti-His (Genescript) (1:2000) in buffer TBSTM (TBST, 0.5% (w/v) skim milk) (1:10 000) for 1 h, at room temperature and, after washing, incubated with the antibody anti-mouse (1:10000) (GE

Healthcare, Buckinghamshire, UK). The detection was performed by degradation of the ECF substrate (GE Healthcare) and detection on a FX apparatus (BioRad, Hercules, EUA).

6.3 Results and Discussion

6.3.1 Production, expression and purification of the recombinant peptide-fusion proteins.

The genes that code for both SP10-5 and Sub5 peptides were fused with the Blad gene to construct the new multifactorial peptide-fusion proteins. The fusion of multiple proteins has already been extensively discussed for biotechnological applications (Elleuche, 2015; Gong *et al*., 2016; Hong *et al*., 2006; Quilis *et al*., 2014; Rothan *et al*., 2014; Trujillo *et al*., 1997; Yu *et al*., 2015). Both genes were joined, in separately, to the C-terminal portion of the Blad gene using a 5-amino acid flexible linker (Gly-Gly-Ser-Gly-Gly). Flexible linkers are usually applied when the joined domains require a certain degree of movement or interaction. The small size of these amino acids provides flexibility, and allows for mobility of the connecting functional domains (Chen *et al*., 2013). The performance of the newly synthetized fusion protein depends largely on the peptide liner that is inserted between the moieties (Klein *et al*., 2014; Li *et al*., 2016; Lu and Feng, 2008; Shamriz *et al*., 2016; Zhao *et al*., 2008). Considering the results obtained in a previous study where recombinant Blad was successfully produced in a soluble form in *E. coli* using MBP (maltose-binding protein) as a solubility enhance partner (unpublished work) the MBP gene was also added to the construction, in the N-terminal end of Blad. MBP has been successfully used has solubility-enhancing partner with great results (Fox *et al*., 2003; Hewitt *et al*., 2011; Kapust and Waugh, 1999), with the additional benefit that it can also be used as affinity tag for purification (Baneyx, 1999; Esposito and Chatterjee, 2006). The design of the peptide-fusion proteins construction is shown in Fig. 1 and the final amino acid sequences are shown in Fig.S1. MBP has a molecular weight of 40.2 kDa, Blad has 20.4 kDa and SP10-5 and Sub5 have 1.5 and 1.7 kDa, respectively. Therefore, the molecular weight of both peptide-fusion proteins was approximately 60 kDa.

Figure 6.1. Production of the recombinant peptide-fusion proteins in *E.coli*. Design of both peptide-fusion proteins.

Both the synthetic genes that code for the newly peptide-fusion proteins, hereby termed His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5, were cloned into pCoofy plasmids, a bacterial expression vector for parallel Sequence and Ligation Independent Cloning (SLIC) containing N-

terminal 6x His and MBP tags (Scholz *et al*., 2013). After induction of over-expression of the genes that code for both peptide-fusion proteins, cells were collected by centrifugation and analyzed by both SDS-PAGE and immunoblot, using an anti-Histag antibody, which demonstrated that the genes that code for both peptide-fusion proteins were being correctly over-expressed and with the expected molecular weight (~60 kDa) (data not shown). To assess the solubility of each of the peptide-fusion protein, both the soluble and insoluble fractions were loaded onto a 12% (w/v) SDS-polyacrilamide gel. Fig. 2 shows that both peptide-fusion proteins were present mainly in the insoluble fraction, under the growth conditions tested.

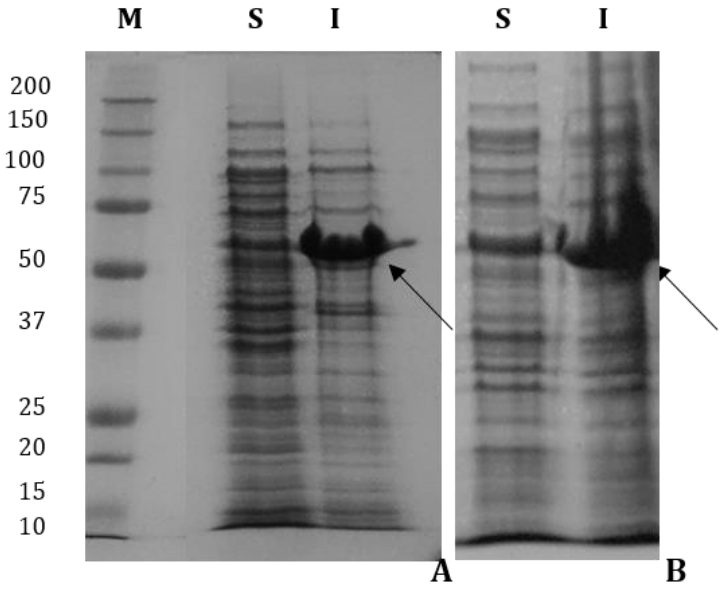


Figure 6.2 SDS-PAGE analysis of both the soluble (**S**) and the insoluble (**I**) fractions resulting from the expression of the fusions His_6-MBP-Blad-SP10-5 (**A**) and His_6-MBP-Blad-Sub5 (**B**). Molecular masses of standards are indicated in kDa.

Another attempt was performed to overcome the insolubility issues. Several expression conditions were tested, including a combination of four different temperatures (20, 25, 30 and 37 °C) with three IPTG concentrations (0.1, 0.5 and 1 mM). After expression under these conditions, both the soluble and insoluble fractions were spotted directly onto a nitrocelulose membrane for dot-blot analysis, using an anti-histag antibody (Fig. 3). The results obtained revealed that the majority of the signal is in the insoluble fraction, despite the temperature or the IPTG concentration tested. The strong signal observed in the insoluble fraction of both peptide-fusion proteins is representative of great levels of expression. However, the fusion

proteins were synthetized as inclusion bodies, meaning that none of the constructs leads to a significant accumulation of the fusion protein in a soluble form. Despite the several evidences that indicate that MBP is a very promising solubility-enhancing partner (Fox *et al*., 2003; Hewitt *et al*., 2011; Kapust and Waugh, 1999), no affinity tag is universally ideal and many of them have the same downside of having different performances with different partner proteins (Esposito and Chatterjee, 2006; Waugh, 2005). Fusing an insoluble protein to a solubility-enhancing partner does not guarantee a soluble fusion protein (Kapust and Waugh, 1999).

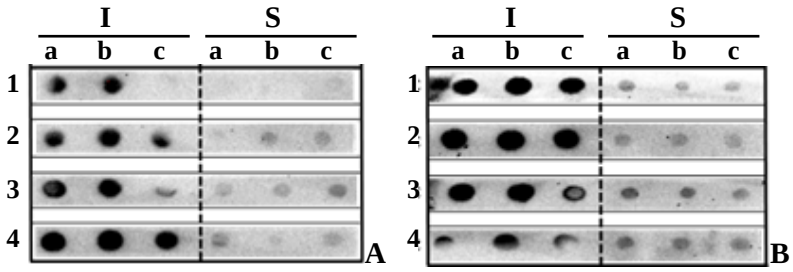


Figure 6.3 Dot-blot analysis of both the soluble (**S**) and insoluble (**I**) fractions resulting from the expression of fusions His6-MBP-Blad-SP10-5 (**A**) and His6-MBP-Blad-Sub5 (**B**) in four different temperatures (1 – 20 °C; 2 – 25 °C; 3 – 30 °C and 4 – 37 °C) with three IPTG concentrations (a – 1 mM, b – 0.5 mM, c – 0.1 mM).

There are multiple factors that contribute towards the formation of protein aggregates during heterologous expression in *E. coli*. In normal circumstances, one protein chain is released from the ribosome of *E.coli* every 35 seconds and the macromolecules concentration can reach as high as 300–400 mg/ml, in these conditions the correct folding of a protein can be considered an extraordinary (Baneyx and Mujacic, 2004). In addition, the newly synthesized recombinant protein is being synthetized in an environment, which may be quite different from that of its original source in terms of pH, osmolarity, redox potential, cofactors, and folding mechanisms (Rosano and Ceccarelli, 2014). Finally, it may also be the case that the insolubility is an intrinsic property of a particular protein (Hewitt *et al*., 2011). Either way, if a given protein fails to rapidly achieve its native conformation it has two possible consequences: partial or complete deposition into inclusion bodies (Baneyx and Mujacic, 2004). A tight control of a variety of parameters like, temperature of expression, expression rate and inducer concentration might help to reduce the formation of aggregates (Esposito and Chatterjee, 2006; Shamriz *et al*., 2016). In many cases, the overexpression of genes in *E.coli* remains an unsolved problem as the proteins continue to be synthetized as inclusion bodies.

Considering the impossibility to produce both fusion proteins in a soluble form, even with the MBP in the construction as solubility enhancing tag, the next step was to solubilize and refold the inclusion bodies, as described on the materials and methods section. The solubilized peptide-fusion proteins were analyzed by SDS-PAGE (Fig.4) and the results obtained revealed that despite both His6-MBP-Blad-SP-10 and His6-MBP-Blad-Sub5 were synthetized as inclusion bodies, and therefore being only soluble in denaturant buffers, it is possible to refold them, by slowly diluting in a renaturing buffer. Moreover, Fig.4 shows that comparing to His6-MBP-Blad-SP10-5, His_6-MBP-Blad-Sub5 is recovered with higher yield, considering the thickness of the respective band.

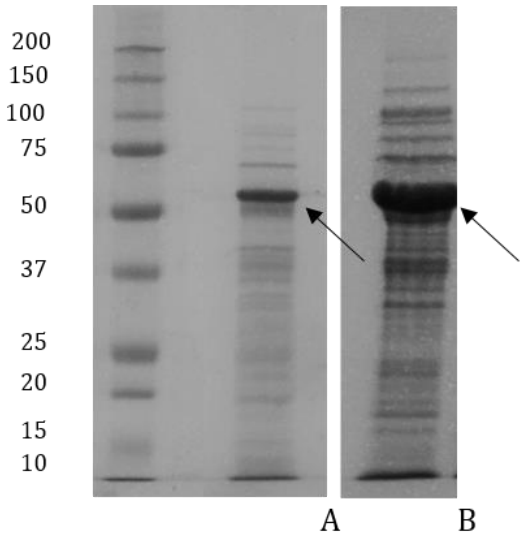


Figure 6.4 SDS-PAGE analysis of the solubilized inclusion bodies of His_6-MBP-Blad-SP10-5 (**A**) and His_6-MBP-Blad-Sub5 (**B**). Molecular masses of standards are indicated in kDa.

The next step was to purify the peptide-fusion proteins. As mentioned above, MBP not only is a powerful solubility enhancing tag, that often helps folding of fusion proteins (Lebendiker and Danieli, 2011) but also can be used as a detection and a purification tag (Liu *et al*., 2009). Considering the addition of the MBP into the constructs, the purification was performed by affinity chromatography, using a MBPTrap™ HP, a column prepacked with dextrin, a resin for which MBP has high affinity. The solubilized peptide-fusion proteins were applied to the prepacked column, washed and eluted with 10 mM maltose in binding buffer and the eluted fractions of both peptide-fusion proteins were analyzed by SDS-PAGE (Fig. S2). The analysis of both the chromatogram and the SDS-PAGE reveals that both fusion proteins were purified with a high purity level, which is in accordance with previous studies that claim that the purification

by capture affinity step on amylose-columns result in a protein that is often 70-90% pure (Khow and Suntrarachun, 2012; Liu *et al*., 2009).

For the proper confirmation of the fusion proteins in study, the corresponding bands were individually cut from the gel and analyzed by LC-MS/MS. The results obtained from mass spectrometry analysis were rather inconclusive. Taking into consideration the specific regions for the peptides SP10-5 and Sub-5 ("LRIIKKILKKLI" and RRWKIVVIRWRR" respectively) it was difficult to obtain a good tryptic peptide for protein identification (trypsin cleaves after lysines and arginines) and thus, be able to identify the recombinant peptides. Both Blad and MBP were positively identified with a 95% confidence level.

Despite the high purity level obtained when purifying both His_6MBPBladSP-10 and His6-MBP-Blad-Sub5 fusion proteins, the yields obtained, considering the need to refold the inclusion bodies by diluting them into a refolding buffer, were rather low. In fact, though formation of inclusion body renders easier protein purification, there is no guarantee that the in vitro refolding will generate large amounts of biologically active products (Needle and Waugh, 2014).

6.3.2 In vitro antimicrobial activity of the fusion proteins. In a new set of experiments, the antimicrobial activity of the fusion proteins His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5 was evaluated and compared with the activity of each individual active compound (SP10-5, Sub-5 and BCO). The results shown in Table 1 reveal that BCO has a potent antifungal activity against both yeasts and filamentous fungi, as expected (Monteiro *et al*., 2015; Pinheiro *et al*., 2016). However, it was also demonstrated that BCO has some antibacterial activity, even though much modest and not against all species. By fusing Blad, the active ingredient of BCO, to a molecule proved to possess biological potential as antibacterial agent (Hilpert *et al*., 2005; Zeitler *et al*., 2013) its spectrum of action should be, theoretically, enhanced.

In a new set of experiments, the antimicrobial activity of the fusion proteins His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5 was evaluated and compared with the activity of each individual active compound (SP10-5, Sub-5 and BCO). BCO has proven antifungal activity against both yeasts and filamentous fungi (Monteiro *et al*., 2015; Pinheiro *et al*., 2016), but lacks antibacterial activity. By fusing Blad, the active ingredient of BCO (Monteiro *et al*., 2010, 2015),

to a molecule proved to possess biological potential as antibacterial agent (Hilpert *et al*., 2005; Zeitler *et al*., 2013) its spectrum of action should be, theoretically, enhanced.

Table 6.1. In vitro susceptibility of various species of bacteria, yeasts and filamentous fungi to the fusion proteins His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5 and BCO as determined by MIC (Minimum Inhibitory Concentration).

Species* (nr. of strains)	MIC (µM) (range)				
	SP10-5	**His6-MBP-Blad-SP10-5**	**Sub5**	**His_6-MBP-Blad-Sub5**	**BCO**
***X. arboricola* (2)**	1.3-5.3	0.065	0.6-2.4	1.032 - 0.516	1.2 - 2.4
***X. vesicatoria* (2)**	1.3-5.3	0.065	0.6-1.2	1.032	2.4
***E. coli* (1)**	> 13	0.516	4.7	NA	NA
***S. typhimurium* (1)**	13	0.258	9.4	NA	NA
***P. aeruginosa* (1)**	> 13	0.516	9.4	NA	NA
***P. syringae* (2)**	1.3	0.065	1.2	1.032	0.6 - 1.2
S. aureus (1)	13	1.032	9.4	1.032	2.4
***C. albicans* (1)**	13	0.032	37.6	0.065	0.074
***C. glabrata* (1)**	42.7	0.065	> 37.6	0.032	0.074
***B. cinerea* (1)**	42.7	0.065	37.6	0.065	0.149

NA – No Activity within the range of concentration tested.

* *B. Botrytis, C. Candida; E. Escherichia, P. Pseudomonas, S. Salmonella, X. Xanthomonas*

Bacterial susceptibility to SP10-alone was variable, but confirmed its potent effect towards *Xanthomonas* spp. and *P. syringae* (Zeitler *et al*., 2013). Although Sub5 is described as having a great antibacterial activity against several species (Ebbensgaard *et al*., 2015; Hilpert *et al*., 2005), in our study MICs were a little bit higher than expected, but still corroborated its efficacy and wide spectrum range. When fused, the activity of SP10-5 (His6-MBP-Blad-SP10-5) was highly enhanced for all bacteria, becoming particularly efficient against *Xanthomonas* spp and *P. syringae*. The most extraordinary results were obtained with *E. coli* and *P. aeruginosa*, as Blad and SP10-5 alone were inactive against these species and the fusion of both was able to inhibit their growth. The same occurred with Sub5 for these species as well as for *S. typhimurium*. Furthermore, the antibacterial activity of the fusion protein His6-MBP-Blad-Sub5 for the

remaining species was always lower than the corresponding antibacterial activity of the individual compounds alone, suggesting a synergistic effect between these molecules. Comparing the antifungal activity of both fusion proteins with that of BCO alone, the construction was able to retain the ability to inhibit the growth of both yeast and filamentous fungi. This was corroborated by the MIC values of Sub-5 and SP10-5 alone for these strains, which were much higher than those observed when fused to Blad but similar to the values of BCO alone, thus confirming that the antifungal activity of the hybrid proteins derives exclusively from Blad. The antimicrobial activity of the isolated MBP was tested and revealed no interferences with the results (data not shown).

Fusion proteins are now being extensively used in the biomedical field as an important tool to detect and purify antibodies (Dong *et al*., 2015) and to design and produce bifunctional enzymes (Tian and Dixon, 2006). In this work, we designed and investigated the antimicrobial activity of the fusion proteins His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5 against important plant pathogens. Both fusions were constructed having the peptides genes fused to the C-terminal portion of the Blad using a 5-amino acid flexible linker (oligopeptide), that is widely used to construct functional fusion proteins. As the name implies, flexible linkers are composed of flexible amino acid residues like glycine, serine and proline which allows the contiguous proteins to move independently (Lee *et al*., 2013).

It is difficult to express antimicrobial proteins in bacteria simply because the active antimicrobial proteins will kill the host. One possible approach is to compel the expression of the target protein as an inclusion body (Haught *et al*., 1998; Lee *et al*., 2000) which, unwittingly, was our case from the beginning. Thus, we were able to express a bactericidal peptide in *E. coli*, without compromising its viability. However, it is well noticed that even after successfully expression of the antimicrobial fusion protein, they constantly present diminished or absent antimicrobial activity (Hongbiao *et al*., 2005). In addition, our results seem to indicate that not all fusion proteins behave equally; when fusing genes that code for two different proteins one can eventually be produced with an impaired bioactivity. Despite the idea that the choice of the component proteins is only based on the desired functions of the fusion protein and that, in most cases, is relatively straightforward (Chen *et al*., 2013), the rationale design of fusion proteins with desired and predictable behavior remains a daunting challenge (Yu *et al*., 2015).

In this work, both constructions retained the ability to inhibit the growth of both yeast and filamentous fungi, and, most importantly, both showed an increased antibacterial activity, probably due to a synergistic effect between Blad and SP10-5 and Sub5. Hongbiao *et al*. (Hongbiao *et al*., 2005) and Lee *et al*. (Lee *et al*., 2013) had already successfully engineered hybrid proteins from two antibacterial peptides. However, the originated fusion proteins presented inferior antibacterial activity than the individual parental ones. In a similar recent study published by Kovalskaya *et al*. (Kovalskaya *et al*., 2011), a hybrid protein, named SAP, was also successfully produced but, in this case, it showed distinct and enhanced biological activities. The results indicate that the performance of the hybrid protein was better or equal to that of the parental proteins. The work reported by Kovalskaya *et al*. (Kovalskaya *et al*., 2011) also demonstrated that the chimeric protein SAP produced in plants of tobacco and potato had a plant protective role against *Colletotrichum coccoides* the casual fungi agent of potato anthracnose and *Clavibacter michiganensis* subsp. *sepedonicus*, which is the bacterium that causes the potato ring rot disease.

Although this proved to be a very interesting study, the ultimate purpose of the authors was to develop a genetic strategy to express the hybrid protein SAP as a self-cleavage protein in plant cells to allow the production of the individual antimicrobial proteins and, in this way, increase the plant pathogen resistance. Our work aimed at generating a new molecule to be used as an active ingredient for plant protection as a plant foliar biopesticide. For a new biopesticide to be of a plant protection interest, a high efficiency needs to be combined with a low toxicity. Although the toxicological profile of the hybrid proteins was not assessed in this study, both the BCO and the individual peptides were previously recognized as low toxic compounds. In the study recently published by Pinheiro *et al*. (Pinheiro *et al*., 2017), the BCO showed no evidence of topical toxicity, genotoxicity and carcinogenicity towards mammalian cells after acute or short-term expositions. In addition, the BCO safety as a plant protection product has been corroborated by all the countries National Regulatory Entities where the product has already been approved. Zeitler *et al*. (Zeitler *et al*., 2013) showed that SP-10 peptide was highly active against a broad spectrum of bacteria, but showed low hemolytic activity and a very low phytotoxicity to plant protoplasts and therefore seem to be well suited as a plant protecting agent. Ebbensgaard *et al*. (Ebbensgaard *et al*., 2015) reported that Sub-5 peptide had no minimal hemolytic activity indicating that it might be safe to use in the concentrations needed to have the antibacterial activity. In conjunction both the high efficacy and the low toxicity

profile allied to a future deeper and focused development of an appropriate formulation may result in a potent protein-based biopesticide for use in agriculture.

The results presented in this study are an important breakthrough and may represent a new approach to design new bi-functional antimicrobial peptides with great potential of being used in medicine, food safety, and agriculture. In conclusion, we designed, engineered and produced fusion proteins with antimicrobial activity against a range of human, spoilage and plant pathogenic bacteria, yeast and filamentous fungus.

6.4 Supplementary material

SP10-5

MKMNKSLIVLCLSAGLLASAPGISLADVNYVPQNTSDAPAIPSAALQQ
LTWTPVDQSKTQTTQLATGGQQLNVPGISGPVAAYSVPANIGELTLTL
TSEVNKQTSVFAPNVLILDQNMTPSAFFPSSYFTYQEPGVMSADRLEG
VMRLTPALGQQKLYVLVFTTEKDLQQTTQLLDPAKAYAKGVGNSIPDI
PDPVARHTTDGLLKLKVKTNSSSSVLVGPLFGSSAPAPVTVGNTAAPA
VAAPAPAPVKKSEPMLNDTESYFNTAIKNAVAKGDVDKALKLLDEAE
RLGSTSARSTFISSVKGKGRRQRNPYHFSSQRFQTLYKNRNGKIRVLERF
DQRTNRLENLQNYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVN
PDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRVVKLAIPINNPGYFYD
FYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRIILGNEDGGSGGLRIIKK
ILKKLI

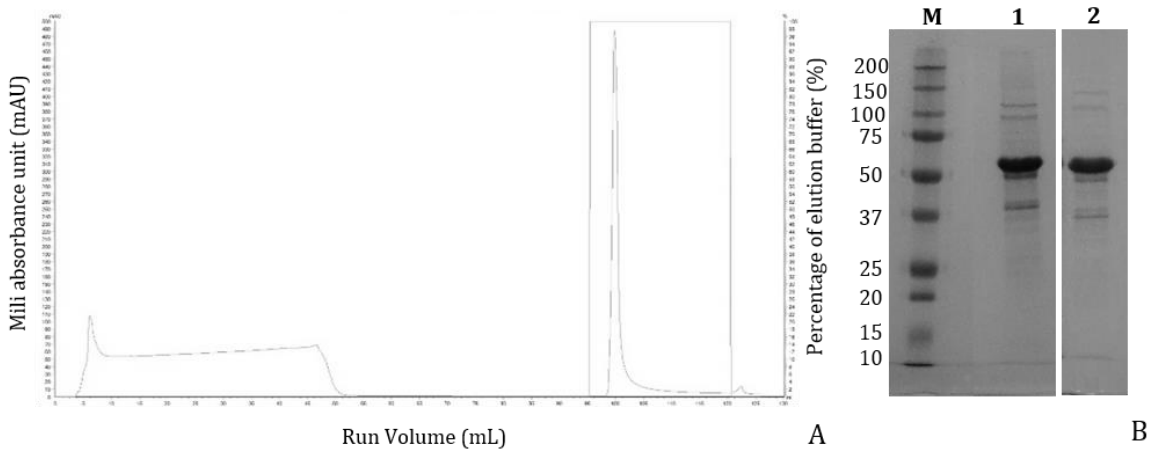
A

Sub5

MKMNKSLIVLCLSAGLLASAPGISLADVNYVPQNTSDAPAIPSAALQQ
LTWTPVDQSKTQTTQLATGGQQLNVPGISGPVAAYSVPANIGELTLTL
TSEVNKQTSVFAPNVLILDQNMTPSAFFPSSYFTYQEPGVMSADRLEG
VMRLTPALGQQKLYVLVFTTEKDLQQTTQLLDPAKAYAKGVGNSIPDI
PDPVARHTTDGLLKLKVKTNSSSSVLVGPLFGSSAPAPVTVGNTAAPA
VAAPAPAPVKKSEPMLNDTESYFNTAIKNAVAKGDVDKALKLLDEAE
RLGSTSARSTFISSVKGKGRRQRNPYHFSSQRFQTLYKNRNGKIRVLERF
DQRTNRLENLQNYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVN
PDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRVVKLAIPINNPGYFYD
FYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRIILGNEDGGSGGRRWKI
VVIRWRR

B

Supplementary Figure S6.1. Amino acid sequences of the peptide-fusion proteins with SP10-5 (**A**) and Sub5 (**B**).



Supplementary Figure S6.2. Chromatogram obtained after purification of the His6MBPBladSP-10 fusion protein by affinity chromatography using a MBPtrap column, eluted with 10 mM maltose in binding buffer (**A**) and SDS-PAGE analysis of the eluted fractions of the peptide-fusion protein His_6-MBP-Blad-SP10-5 and His_6-MBP-Blad-Sub5 (lanes 1 and 2, respectively) (**B**). Molecular masses of standards are indicated in kDa.

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Chapter 7

Final Remarks

Chapter 7 - Final Remarks

BCO-based treatment seems to be a truly remarkable solution to fungal infections, as it appears to fulfill all the prerequisites to function as a new paradigm or even an archetype for the future of pathogenic fungal control. In summary, the overall results of this work provided the necessary data to disclose BCO biochemical characteristics, its potent antifungal activity against an array of yeast and filamentous fungi, its low toxicological profile and its highly complex and multitarget mode of action. Moreover, Blad was successfully fused to an antibacterial peptide, leading to the development of a chimeric bifunctional protein.

The strong antifungal activity of BCO against a variety of plant and animal pathogenic fungi here demonstrated along with its extreme resistance to denaturation and absence of toxicity to both mammals and pollinators, confer great potential as an antifungal compound for agricultural and clinical applications. In fact, BCO is the active ingredient of the agricultural fungicide Fracture®, which is already on sale in some countries (USA, Canada, and South Korea), and will soon be available in others jurisdictions (Europe, China and Australia). According to our results, Blad polypeptide has also potential to be applied in combination with an antibacterial peptide, SP10-5, through the design and production of a fusion protein, named "super-Blad" – a bifunctional protein that possesses both antifungal and antibacterial activities. This designed drug can be considered a very promising compound for use in plant protection although a deeper and focused development of an appropriate formulation is still required. If it succeeds, the chimeric drug may result in a potent biopesticide which can replace *per se* two conventional chemistries, with less impact on the environment.

This work also played a major role in elucidating BCO's mode of action. BCO has the ability to cross the cell membrane in an ergosterol-independent manner, compromising its integrity, but without causing visible rupture of the cell wall. The exact mechanism by which BCO enters the cell is yet to be elucidated, however, once inside the cells, it causes several ultrastructural alterations. The adverse intra and/or extracellular environment imposed by BCO is a result of what we considered to be its primary mode of action – a metal scavenging activity, with a particular effect on zinc and iron, shattering cell homeostasis. As a result, an oxidative stress is generated inside the cells which, ultimately, culminate in an apoptotic cell death. The discovery of this new and multitarget mode of action, unlikely to promote resistance, led the Fungicide Resistance Action Committee (FRAC) to include BCO in a new category, M12, of the 2016 issue

of its Code List©. In this way, BCO separates itself from all other fungicides with unknown cross-resistance with any of other fungicide class, making it a unique active ingredient in disease management programs.

From the clinical point of view, and as a first approach, BCO was evaluated as a potential topical fungicide. The results presented here are very promising, as BCO showed no evidence of topical toxicity towards mammalian cells after acute or short-term expositions. Further work is now required to develop a viable and effective formulation for the treatment of topical mycoses. Other studies should also be conducted to determine its potential for the treatment of systemic fungal infections, namely its interaction with fungal cells (e.g. its mode of cell entrance), and with the human body. To address this, the antifungal activity should be studied both *in vitro*, focusing its interference in the interaction fungal-cells/host-cells in model cell lines such as phagocytic-macrophage derived cell line and a non-phagocytic cell line, and *in vivo*, in a model of disseminated mycosis on mice. These results will provide a measure of the efficacy of BCO in the prevention and treatment of systemic fungal diseases.

Lastly, in an attempt to perform a deep structural characterization, Blad was produced in a recombinant form by heterologous expression. However, the results were not conclusive due to a host protein contamination. Giving that the knowledge of the three-dimensional structure of Blad could be a valuable asset to better understand the mode of action of BCO, a different approach must be followed, in the future, to allow the proper production of recombinant Blad.